Complete Regression of Established Human Glioblastoma Tumor Xenograft by Interleukin-4 Toxin Therapy

Syed R. Husain, Niti Behari, Robert J. Kreitman, Ira Pastan, and Raj K. Puri

ABSTRACT

No curative therapy is available for malignant gliomas. We have discovered that human glioblastoma cells express high affinity interleukin-4 receptor (IL-4R), which is an attractive target for receptor-directed IL-4 toxicity. The IL-4 toxin, IL-4(38-37)-PE38KDEL, is a fusion protein containing translocation and enzymatic domains of Pseudomonas exotoxin and a circularly permuted human IL-4. The IL-4 toxin binds specifically to the IL-4R and is highly cytotoxic to glioblastoma cells, as determined by clonogenic and protein synthesis inhibition assays. Intratumoral administration of the IL-4 toxin given on alternate days for 3–4 doses into U251 glioblastoma flank tumors in nude mice, showed a complete remission of small (~13 mm³) and large (~60 mm³) tumors in all animals, without any evidence of toxicity. A significant antitumor activity was also observed when the IL-4 toxin was administered via i.p. and i.v. routes. These results demonstrate that the IL-4 toxin may be a new therapeutic drug for the treatment of human glioblastoma. Therefore, we have begun a Phase I clinical trial with IL-4(38-37)-PE38KDEL for treatment of human glioblastoma.

INTRODUCTION

Primary malignant brain tumors are diagnosed in approximately 17,500 adult Americans each year. The most common brain tumors are of glial cell origin. Glioblastoma multiforme, one of the most malignant forms of gliomas, constitutes at least 35% of all primary brain tumors (1, 2). Despite aggressive multimodality therapy, the prognosis of malignant gliomas remains dismal and is almost uniformly fatal. The malignant gliomas continue to present a challenge to define effective modalities of treatment. The possible role of immunotherapy in the management of glioblastoma is still under investigation (3). Research efforts have focused on innovative techniques to allow selective targeting of receptors expressed on tumor cells, thereby minimizing dose-limiting side effects and maximizing therapeutic efficacy (4–6). We recently reported that human glioblastoma cells express receptors for IL-4 that can be effectively targeted in vitro with a PE-based IL-4 chimeric protein, termed IL-4-PE(15) (7). Later, a circularly permuted chimeric toxin, IL-4(38-37)-PE38KDEL, was prepared to manifest improved cytotoxicity against various IL-4R-bearing tumor cells (8–10). In IL-4(38-37)-PE38KDEL, the circularly permuted ligand consists of IL-4 amino acids 38–129 fused via the linker GGNG to IL-4 amino acids 1–37, which in turn are fused to the truncated form of PE. This toxin binds with higher affinity than previous IL-4 toxins and induces more potent cell killing (11).

Because previous studies have demonstrated a lack of IL-4R expression on normal brain tissues (11), it is believed that a therapeutic window exists between the expression of the IL-4R on brain tumor cells compared with normal brain tissues. This differential IL-4R expression may be exploited by targeted toxin therapy. To assess in vivo antitumor activity of IL-4(38-37)-PE38KDEL, human glioblastoma (U251) cells were implanted s.c. in athymic nude mice and treated with the IL-4 toxin administered via different routes.

MATERIALS AND METHODS

Cell Culture and Reagents. A U251 glioblastoma cell line was obtained from the National Cancer Institute/Frederick Cancer Research Facility, Division of Cancer Treatment Tumor Repository (Frederick, MD). The cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Biowhitaker, Walkersville, MD), 3 mM L-glutamine, and 50 μg/ml gentamicin (Biowhitaker).

Recombinant IL-4 Toxin. The chimeric IL-4(38-37)-PE38KDEL toxin was prepared by fusing a truncated PE gene encoding PE38KDEL 3' to a circularly permuted IL-4 mutant gene encoding IL-4 amino acids 38–129, the linker GGNG, and IL-4 amino acids 1–37. The resulting chimeric protein was termed as IL-4 (38–37)-PE38KDEL, as described previously (8).

Clonogenic Assay. The in vitro activity of IL-4(38-37)-PE38KDEL on U251 cells was determined by colony-forming assay (12). The cells were plated in triplicates in 100-mm Petri dishes with 7 ml of medium and were allowed to attach for 20–22 h. The number of cells/plate was chosen such that more than 100 colonies were obtained in the control group. The cells were exposed to different concentrations of toxin (~100 ng/ml) for 10 days at 37°C in a humidified incubator. The cells were washed, fixed, and stained with crystal violet (0.25% in 25% alcohol). The colonies consisting of more than 50 cells were scored. In another experiment, the cells were treated with a fixed concentration of toxin (20 ng/ml) for various periods of time. After treatment, the dishes were washed and cultured with drug-free medium for 10 days. The percentage of colony survival was determined from the colonies formed in the control and treated groups.

Protein Synthesis Inhibition Assay. The in vitro cytotoxicity of the IL-4 toxin, IL-4(38-37)-PE38KDEL, was measured by inhibition of protein synthesis. Protein synthesis was determined by the incorporation of [3H]Leucine into human glioblastoma cells, as described earlier (9, 10, 13). In brief, 1 × 10⁴ cells/well were cultured overnight in 96-well flat-bottomed microtiter plates. The medium was aspirated and replaced by 200 μl of leucine-free medium (Biofluids, Inc., Rockville, MD) with or without various concentrations of IL-4(38-37)-PE38KDEL. For blocking experiments, cells were preincubated into human glioblastoma cells, as described earlier (9, 10, 13). In brief, 1 × 10⁴ cells/well were incubated overnight in 96-well flat-bottomed microtiter plates. The medium was aspirated and replaced by 200 μl of leucine-free medium (Biofluids, Inc., Rockville, MD) with or without various concentrations of IL-4(38-37)-PE38KDEL. For blocking experiments, cells were preincubated with IL-4 for 45 min at 37°C before the addition of the IL-4 toxin to the cells. Cells were further incubated for 20–24 h at 37°C, and then 1 μCi of [3H]Leucine (NEN, Boston, MA) was added to each well and cultured for an additional 4 h. The cells were washed and harvested on fiberglass filtermat and the cell-associated radioactivity was measured with a Beta Plate Counter (Wallac, Gaithersburg, MD). The data were obtained from the average of quadruplicates, and the assays were repeated several times. The concentration of IL-4(38-37)-PE38KDEL at which 50% inhibition of protein synthesis (IC₅₀) occurred, was calculated.

Antitumor Activity of IL-4 Toxin in Nude Mice Implanted with Human Glioblastoma. Female athymic nude mice, 4 weeks of age (~20 g), were obtained from the Frederick Cancer Center Animal Facilities (Frederick, MD). Animals were housed in filter-top cages in a laminar flow hood. Human glioblastoma tumors were established in nude mice by s.c. injection of 2 × 10⁶ U251 cells in 100 μl of PBS plus 0.2% HSA, into the flank. Palpable tumors developed within 3–6 days. The mice then received injections of excipient or chimeric toxin with a 22-gauge needle either i.v. (100 μl), i.p.
(500 µl), or IT (20 µl), as indicated in the figure legends. For IT administration, IL-4(38–37)-PE38KDEL was injected slowly (10 µl/min) in a total volume of 20 µl of PBS containing 0.2% HSA into the left and right sides of tumor (10 µl at each side), at each day of injection. Tumor volumes were calculated based on the formula: Volume = (length) x (width)²/2 (0.4).

RESULTS

Our laboratory has focused on the expression and function of the IL-4R on human tumors, and targeting these receptors with PE-based chimeric toxins. In pursuit of our objective, we have recently identified that human brain tumor cells express high numbers of high affinity IL-4R with Kd of ~100 pM (7, 11). Glioblastoma cell lines stained strongly with anti-IL-4R antibody and Northern analysis revealed that these cells expressed mRNA for IL-4R p140 (data not shown).

Using the glioblastoma cell line U251, which expresses the IL-4R and can grow as a solid tumor in athymic nude mice (6), we investigated whether the IL-4R could be targeted in vivo by a chimeric cytotoxic protein composed of a circularly permuted IL-4 and a mutated form of PE (8–12). We compared the cytotoxicity of different IL-4 toxins on glioblastoma cells by the measurement of the inhibition of protein synthesis (determined by the incorporation of [3H]Leucine). We showed that the circularly permuted IL-4 toxin, IL-4(38–37)-PE38KDEL, was effective particularly with U251 and T98G glioblastomas (11). The IC₅₀ observed for U251 cells was 4.5 ng/ml, which was in very close agreement with our previous report (6.5 ± 1.3 ng/ml; Ref. 11). The cytotoxic activity of IL-4(38–37)-PE38KDEL was completely blocked by an excess of IL-4, indicating that cytotoxicity mediated by the IL-4 toxin is specific (11, 12). The cytotoxicity of IL-4(38–37)-PE38KDEL was also tested on normal human bone marrow-derived cells, EBV-immortalized B cells, promonocytic (U937) cells, H9 T cells, and normal endothelial cells. Consistent with the expression of low numbers of IL-4Rs, the IL-4 toxin was either not cytotoxic or was only slightly cytotoxic on these resting human cells (10, 12).

In vitro clonogenic assays revealed that IL-4(38–37)-PE38KDEL has marked inhibitory activity in colony formation in U251 cells in a concentration-dependent manner (Table 1). Complete inhibition of colony formation was observed at 20 ng/ml IL-4 toxin. The IC₅₀ of IL-4(38–37)-PE38KDEL by clonogenic assay (IC₅₀ = 1.0 ng/ml) was slightly lower than the IC₅₀ determined by protein synthesis inhibition (4.5 ng/ml). To determine how long an exposure to IL-4(38–37)-PE38KDEL was required for optimal cytotoxic effects, microcolonies of two glioblastoma cell lines (U251 and T98G) were incubated with a fixed concentration of IL-4(38–37)-PE38KDEL (20 ng/ml) for different periods of time (Fig. 1A and B). The IL-4 toxin exhibited its cytotoxic effect in a time-dependent fashion. More than 91% of U251 colonies (Fig. 1A) were killed within 4 h, and 86% of T98G colonies (Fig. 1B) were killed within 24 h. Continuous exposure to the IL-4 toxin for 10 days completely inhibited colony formation in both cell lines (data not shown).

Efficacy of IL-4(38–37)-PE38KDEL in Nude Mice Implanted with Human Glioblastoma. In vitro data indicated that glioblastoma cells, including U251, are very sensitive to the IL-4 toxin. However, whether glioblastoma cells are sensitive in vivo is not known. To assess in vivo activity of the toxin, nude mice, 4 weeks of age, received s.c. injections of 2–4 x 10⁶ U251 glioblastoma cells, resulting in the development of tumors with a mean size of 13–60 mm³ by days 3–6. U251 cells consistently generated solid tumors in all animals that received injections. The efficacy of IL-4(38–37)-PE38KDEL, when administered by different routes and dosing schedules, was evaluated in these mice.

i.p. Treatment. i.p. administration with 25, 50, or 100 µg/kg/dose of IL-4(38–37)-PE38KDEL on QOD for a total of three doses significantly inhibited U251 tumor growth in a dose-dependent fashion (Fig. 2A). The first injection, given 4 days after tumor implantation (day 4), resulted in a 90% reduction in mean tumor size by day 6 at 50 and 100 µg/kg doses. After the third injection (day 8), the established tumors had regressed completely in both dose groups. The animals remained tumor-free for 28 days. Tumor growth (3 mm³) was observed in one of five mice dosed at 100 µg/kg, but most of the tumor was necrotic. After 32 days, a small tumor (3 mm³) appeared in one animal, each dosed at 50 and 100 µg/kg. By day 43, an additional

Table 1 Inhibition of U251 cell growth by IL-4(38–37)-PE38KDEL in a clonogenic assay

<table>
<thead>
<tr>
<th>IL-4(38–37)-PE38KDEL (ng/ml)</th>
<th>Colonies ± SD (% of control)</th>
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<tr>
<td>1</td>
<td>49.1 ± 9.1</td>
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<tr>
<td>5</td>
<td>7.3 ± 1.9</td>
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<td>10</td>
<td>4.0 ± 2.8</td>
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Fig. 1. Time-dependent in vitro inhibition of glioblastoma cell growth by IL-4(38–37)-PE38KDEL in a clonogenic assay. U251 (A) or T98G (B) cells were plated in triplicate on Petri dishes and treated with IL-4(38–37)-PE38KDEL (20 ng/ml) on the subsequent day. The adhered cells were washed at the indicated times and replenished with fresh medium and cultured for a total of 10 days. The colonies formed were washed and stained with crystal violet. The colonies were counted, and the data were plotted against the time of exposure. The experiments were repeated at least two times at different occasions. The data shown are the mean ± SD of triplicate plates set for each time point.
animal in each of these groups showed tumor recurrence (mean size of about 10 mm³).

Treatment with a lower dose of IL-4(38–37)-PE38KDEL (25 μg/kg), QOD x 3 doses, also resulted in tumor regression, with a 50% reduction in tumor size by day 8 (the last day of treatment). The tumor grew slowly thereafter, and mean sizes were significantly lower compared with exponentially growing tumors in the control group. A higher dose of 200 μg/kg, QOD x 3 doses, of IL-4(38–37)-PE38KDEL showed antitumor activity comparable to 50 and 100 μg/kg doses (data not shown). Thus, a dose-response effect was seen with the maximal response being complete regression in some animals.

All animals were sacrificed on day 47. The animals tolerated the therapy well, with no signs of apparent toxicity or deaths.

To understand the mechanism of incomplete antitumor response after i.p. administration of IL-4(38–37)-PE38KDEL, tumors that occurred after treatment were excised, minced, and digested with collagenase, hyaluronidase, and DNase enzymes to obtain a single cell suspension. After passing through seven passages, the sensitivity of cells toward IL-4(38–37)-PE38KDEL were tested by protein synthesis inhibition assay and compared with the IC₅₀ of the U251 cell line. The cells from treated tumors were found to be as sensitive as the cells used to implant the tumors (data not shown). This experiment demonstrated that the glioma cells did not acquire the resistance in vivo against IL-4(38–37)-PE38KDEL due to selection of a stable subclone expressing low IL-4R levels or some other attribute that would allow escape from the IL-4 toxin treatment, whereas it is possible that glioma cells were transiently resistant to the IL-4 toxin. It is also likely and we favor most that the IL-4 toxin was not thoroughly distributed in tumor bed, which allowed the escape of some tumor cells resulting in regrowth.

**i.v. Treatment.** When s.c. tumors reached 45 mm³, mice received i.v. injections of 1, 2 and 4 μg (representing 50, 100, or 200 μg/kg, respectively) of circularly permuted IL-4 toxin on QOD for a total of 3 doses, tumor regression was noted in 100% of the IL-4(38–37)-PE38KDEL-treated mice, irrespective of the dose administered (Fig. 2B), beginning as early as day 6. As shown in this figure, the effect of the IL-4 toxin was clearly dose-dependent. By day 10, optimum regression of an established tumor was noted in animals treated with the highest dose of circularly permuted IL-4 toxin. The tumors in the control mice, who were injected with excipient (0.2% HSA in PBS) alone, continued exponential tumor growth. After day 10, the tumor in all groups began to increase, but tumor size always remained significantly lower in the IL-4(38–37)-PE38KDEL-treated animals compared with the control animals, through day 42.

**IT Treatment.** To determine whether IT treatment would lead to improved antitumor activity, nude mice with s.c. U251 tumors (mean size 13 mm³) received IT injections of a total IL-4 toxin dose of up to 1000 μg/kg, administered by various dosing schedules (Fig. 2C). The treatment began 6 days after tumor implantation (day 6). Tumors in the mice treated with 250 μg/kg IL-4 toxin, QOD x 3 doses, began to decrease significantly in size by day 10, with three of four mice exhibiting complete regression by day 14. By day 24, 100% of the mice (four of four) were tumor-free. Although tumors reoccurred in...
two of four mice by day 31 (37 days from tumor implant), the mean size of the tumors remained significantly smaller than the control group (2% of the control tumor size, or 20 mm³ versus 1135 mm³) through day 58.

All four mice treated with 250 μg/kg IL-4 toxin, QOD × 4 doses (days 6, 8, 10, and 12), showed complete response by day 12 and remained tumor-free for a total of 64 days (Fig. 2C). One mouse displayed tumor growth (about 3 mm³), which increased very slowly thereafter and reached a size of 26 mm³ by the end of the experiment (91 days). The control mice were sacrificed on day 76 because of heavy tumor burden, in accordance with the guidelines of the NIH Animal Research Advisory Committee. The IL-4 toxin-treated animals were monitored for a total of 91 days, and all complete responders remained disease-free (Fig. 2C).

A similar treatment with 750 μg/kg × 1 dose of IL-4(38-37)-PE38KDEL exhibited complete regression of tumors in all four treated mice by day 14, and they remained tumor-free through 31 days. Thereafter, one mouse began to show evidence of tumor. Another mouse in the same group displayed tumor growth on day 45. By day 70, the tumors in both mice reached a size of 400 and 147 mm³, respectively. The other two mice remained completely tumor-free until the end of the experiment (day 91). The control group had a tumor size of 1940 mm³ on day 70.

On the basis of the promising results, we evaluated the antitumor activity of IL-4(38-37)-PE38KDEL against relatively larger tumors of 60 mm³ size. s.c. implantation of 4 × 10⁶ U251 cells yielded large tumors by day 4. Treatment with 750 μg/kg IL-4 toxin was initiated on day 4, with additional doses administered on days 6 and 8 (Fig. 3A). All treated animals (n = 6) showed substantial tumor regression as early as day 6. Complete tumor regression in 100% of the mice was observed by the day of the last injection (day 8). These mice remained tumor-free for over 100 days. All control animals (n = 7) that received injections of excipient quickly developed tumors and were sacrificed by day 60, before ulceration of the progressively growing tumors had occurred.

To assess the distribution of IL-4(38-37)-PE38KDEL after IT injection as it relates to its antitumor activity, we implanted tumors in both flanks of each nude mouse (n = 7). The tumors in the left flank were injected IT with 750 μg/kg, QOD × 3 doses of IL-4 toxin (Fig. 3B), and tumors in the right flank received excipient solution. Additional mice received excipient only IT in both flanks (designated as control). On the day of the third injection (day 7), the IL-4 toxin-treated tumors in the left flank were completely regressed in all animals (n = 7), and these flanks remained tumor-free until termination of the study (day 44). However, excipient-treated flank tumors in the right flank continued to grow in all mice at a rate comparable with the control mice.

**DISCUSSION**

Recombinant toxins are bifunctional proteins that comprise a tumor-cell-specific recognition domain and an enzymatic toxin domain. Immunoconjugates/cytotoxins have shown promising activity both in cell killing as depicted in in vitro assays and in regressing human tumor growth, in some xenograft models (4, 14-18). We have identified a potentially useful cell recognition target in the form of IL-4Rs in human malignant astrocytoma (glioblastoma). By reverse transcription-PCR, we have previously reported that 76% of the 21 biopsy samples were positive for IL-4R expression (11). Recently, we also tested tissue sections from seven biopsy samples of human brain tumors (e.g., meningioma, oligodendroglioma, pituitary adenoma, astrocytoma, hemangioblastoma, and glioblastoma multiforme). All of these sections were positive for IL-4R expression by immunohistochemistry.

In vivo activity of cytotoxins recognizing IL-4Rs (7-12) has been tested in nude mice implanted with A431, an epidermoid carcinoma cell line (9). Here, we report that i.v., i.p., or IT administration of circularly permuted IL-4 toxin induced growth arrest and regression of established glioblastoma tumors. Nude mice received injections of U251 cells (3–4 × 10⁶) s.c. on day 0. A, tumors (~60 mm³) were injected IT with the IL-4 toxin or excipient on days 4, 6, and 8 in a similar manner as described for Fig. 2C. B, tumors were implanted on both flanks of mice and treatment began on day 4 with IT injection of 20 μl of either excipient or 750 μg/kg IL-4(38-37)-PE38KDEL on QOD for a total of three doses. The • and ○ represent the tumors in each flank of the control group that received excipient only. The groups were treated with IL-4(38-37)-PE38KDEL in the left flank only (•) or treated with excipient in the right flank only (○). The data is presented as the tumor size at day 70. The error bars represent the SD in tumor sizes of 6-7 animals in each group.

R. K. Puri et al. unpublished observations.
toxin up to 200 μg/kg, QOD × 3, also inhibited tumor growth, but total regression was not achieved. This incomplete response is most likely due to the short half-life of the IL-4 toxin in blood (9), as well as the low doses administered in this study. It is also possible that some tumor cells may have generated transient resistance, which escaped initial cell death. We have previously observed that a higher systemic dose of IL-4 toxin (>400 μg/kg, QOD × 3) caused consistent, reversible hepatic changes including hepatocyte swelling, vacuolization, and regeneration (9, 11). In an attempt to avoid these hepatic toxicities as well as achieve the desired antitumor dose, we sought to deliver the IL-4 toxin locally. IT injection of 750 μg/kg, QOD × 3 doses of IL-4(38–37)-PE38KDEL into large established tumors (60 mm³) resulted in a total regression in 100% of the nude mice bearing human glioblastoma xenograft. After discontinuation of the treatment, the mice remained tumor-free for at least 100 days, and no tumor recurrence was detected upon termination of the studies.

When only one tumor was treated in mice bearing bilateral tumors located on the flank, the effect was restricted to the toxin-injected tumor, suggesting that the toxin remained localized to the tumor bed and did not escape in sufficient quantity to the systemic circulation to cause regression of contralateral tumors or cause nonspecific toxicity in animals.

The preclinical toxicity evaluation of this circularly permuted IL-4 toxin has also recently been performed by our group using Cynomolgus monkeys and rats (11). By local intrathecal administration, high levels of IL-4(38–37)-PE38KDEL could be achieved without any apparent adverse effects on the central nervous system, as well as on other major organ systems. In addition, serum analysis revealed the absence of the IL-4 toxin in the monkeys studied. Doses up to 6 μg/kg IL-4(38–37)-PE38KDEL were not detrimental to normal brain tissues, explained by the observation that normal brain cells do not express significant levels of IL-4R (11). The experimental data reported here successfully demonstrate that administration of the IL-4 toxin to a nude mouse xenograft tumor model results in significant antitumor activity against human glioblastoma without any evidence of nonspecific toxicity. IT injection of the IL-4 toxin most likely resulted in saturation of tumor bed, with subsequent complete eradication of established tumors. Thus, it is reasonable to believe that intraglioma administration of IL-4(38–37)-PE38KDEL will saturate the entire tumor bed and, due to bulk flow action (19–22), this toxin may also be able to saturate the immediate proximity of the bed into which tumor cells have escaped causing recurrence of lethal disease. However, we have not addressed the complexity of delivery technique and heterogeneity of tumors in this study. Human gliomas can be quite irregular and heterogenous with areas of varying cellularity and vascularity, and the presence or absence of necrosis. We have also not addressed whether the IL-4 toxin protein can penetrate the blood brain barrier when administered systemically. However, one can take an advantage of direct IT therapy for brain tumors, which will bypass a blood brain barrier and deliver high local concentration of the drug. High-flow microinfusion techniques have been developed that have shown distribution of high- and low-molecular weight proteins in targeted areas of the brain (19–22). Therefore, we envision to saturate a whole tumor bed with the IL-4 toxin over a period of days by slow IT administration using multiple catheters.

On the basis of our current results as well as previous studies, we have begun a Phase I clinical trial for the treatment of recurrent glioblastoma by IT administration of IL-4 toxin. Because IT administration surpasses dose-limiting disadvantages that may be associated with systemic exposure, and because glioblastoma multiforme is not generally a systemic disease, IT injection of IL-4(38–37)-PE38KDEL offers a promising new treatment for brain tumors and its clinical results are awaited.

ACKNOWLEDGMENTS

We thank Mrs. Pamela Dover for excellent technical assistance; Ms. Meredith Sarabian and Drs. Nick Obiri and Robert Rand for helpful suggestions and reading this manuscript; and Dr. Frederick Varriacchio for immunohistochemistry.

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