

A safety and efficacy study of local delivery of interleukin-12 transgene by PPC polymer in a model of experimental glioma

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Interleukin-12 (IL-12) triggers an antitumoral immune response and an antiangiogenic effect against cancer. In this study, we tested a novel polymeric vehicle for IL-12 gene therapy along with adjuvant local biodegradable carmustine (BCNU) chemotherapy for the treatment of malignant glioma. Highly concentrated DNA/PPC (polyethylenimine covalently modified with methoxypolyethyleneglycol and cholesterol) complexes were used to deliver a murine plasmid encoding IL-12 (pmIL-12). For toxicity assessment, mice received intracranial injections with different volumes of pmIL-12/PPC. For efficacy, mice with intracranial GL261 glioma were treated with local delivery of pmIL-12/PPC and/or BCNU-containing polymers. Intracranial injections of 5–10 μ l of pmIL-12/PPC were well tolerated and led to IL-12 expression in the brains of treated animals. Treatment with pmIL-12/PPC led to a significant increase in survival compared with untreated mice (median survival 57 days; 25% long-term survival >95 vs. 45 days for control; $P < 0.05$). Treatment with BCNU led to a significant increase in survival compared with untreated mice, with 75% of treated mice having a long-term survival >95 days,

($P < 0.05$). Most importantly, the combination of BCNU and pmIL-12/PPC led to a survival of 100% of the mice for 95 days after treatment ($P < 0.0001$). This novel strategy is safe and effective for the treatment of malignant glioma. The synergy resultant from the combination of locally administered pmIL-12/PPC and BCNU suggests a role for this approach in the treatment of malignant brain tumors. *Anti-Cancer Drugs* 19:133–142 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

High-grade gliomas represent the most common primary malignant tumor of the adult central nervous system, and, unfortunately, the one with the worst prognosis. The aggressive nature of this neoplasia is closely related to its complex pathophysiology. In particular, evasion of the immune system by gliomas limits an effective antitumoral response. Indeed, the cellular immune response of patients and animals with gliomas is impaired [1–3]. The first reports described a lack of proliferation of the peripheral T-cells of these patients after exposure to phytohemagglutinin [1,2]. This phenomenon has been partially explained by the immunosuppressive actions of infiltrating CD25⁺ CD4⁺ T-regulatory cells [4–12]. Other factors that contribute to the immunosuppressive status include the secretion of transforming growth factor (TGF- β) [13–15] and prostaglandins by tumor cells [16,17]. In addition, the decreased level of major histocompatibility complex class I expression by gliomas [18,19] and the expression of human leukocyte antigen (HLA)-G, a nontypical class I MHC molecule [20], seem to play a role as well [21].

New therapies are being designed to maximize antitumor effects while minimizing the toxicities associated with treatment. Some of the experimental treatments are based on immunotherapy, stem-cell therapy, local chemotherapy and radiotherapy [22–27]. In addition, gene therapy is becoming a promising therapeutic alternative. Indeed, the fact that brain tumors do not present with metastases outside the central nervous system allows for local delivery of vectors carrying therapeutic genes [28,29]. To achieve a robust transgene expression and limited toxicity, a wide variety of delivery systems are being developed and tested. Vehicles vary and include stem cells, viral vectors, liposomes and synthetic polymers.

In the context of immunotherapy for gliomas, interleukin-12 (IL-12) is appealing for a number of reasons. IL-12 is a cytokine with strong antitumor properties mediated through the induction of immunostimulatory mechanisms, including T-lymphocyte and natural killer cell activation and secretion of interferon- γ (IFN- γ) from cytotoxic lymphocytes [30–34]. As an immunotherapeutic agent, IL-12 has proven to elicit an effective immunity

leading to tumor regression in animal models [35–37]. Moreover, clinical responses to recombinant human IL-12, administered by intravenous, subcutaneous or intraperitoneal injection, have been observed in patients with renal cell carcinoma, melanoma, cutaneous T-cell lymphoma, and peritoneal metastasis from ovarian cancer [38–44]. In addition to its immune-related features, IL-12 also has significant antiangiogenic properties. The latter characteristic has been attributed to elevations of IFN- γ and interferon-inducible protein 10 (IP-10), as well as inhibition of vascular endothelial growth factor secondary to IL-12 stimulation [45,46].

IL-12 has already been tested for the treatment of brain tumors in preclinical and human studies [47–55]. For instance, local intracranial delivery of nonreplicating 9L cells, genetically engineered to produce IL-12, was proven effective in a rat glioma model [48]. Subsequently, other groups [47,49–54] have also shown that local delivery of IL-12, either via cells or viral vectors, significantly prolongs the survival of mice and rats bearing brain tumors. As IL-12 provides both immunomodulation and a strong antiangiogenic effect, the efficacy shown in these early preclinical studies provides a compelling rationale for the further development of this immune-based therapy.

In the context of gene therapy vectors, novel polymer-based gene delivery systems seem to improve upon the safety and manufacturing issues associated with viral vectors [56–58]. For this reason, we tested one such polymer delivery system composed of a low-molecular-weight, branched polyethylenimine (PEI), which was covalently linked with functional groups methoxypolyethyleneglycol (mPEG) and cholesterol (CHOL), termed PPC [59]. Injection of murine IL-12 plasmid (pmIL-12) formulated with PPC into mice bearing tumors resulted in significant inhibition of neoplastic growth in various solid tumors and peritoneally disseminated cancers [59]. PPC polymer has also been shown to have very low toxicity in animals and is currently being evaluated for local gene delivery of IL-12 in recurrent ovarian cancer patients. To date, no dose-limiting toxicity has been reported.

In order to be used effectively for brain tumors, the plasmid must be formulated at very high concentrations in order to achieve therapeutically relevant delivery in small volume spaces. In this study, we generated a highly concentrated nanopharmaceutical comprising an IL-12 plasmid and a novel polymeric gene carrier (PPC). We assessed the biodistribution and safety of this system after intracranial administration. In addition, we evaluated the therapeutic effect and characterized the immune response associated with IL-12 expression in a murine model of glioma. To further explore its potential application as an adjuvant therapy in the clinical setting,

we tested the PPC/IL-12 system in combination with biodegradable carmustine (BCNU), a chemotherapeutic agent already approved by the US Food and Drug Administration (FDA) for the treatment of malignant glioma [60,61].

Materials and methods

Animals

Six- to 8- week-old C57BL/6 male mice were obtained from Taconic Laboratory (Hudson, New York, USA). Mice were housed and maintained under pathogen-free conditions in accordance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Chicago. Six animals per group were used for toxicity and 10 animals per group were used for efficacy studies.

Synthesis and analysis of physical/chemical properties of polyethylenimine covalently modified with methoxypolyethyleneglycol and cholesterol

PPC was synthesized by combining branched PEI (BPEI, MW 1800), purchased from Polysciences Inc. (Warrington, Pennsylvania, USA), with cholesteryl chloroformate (Aldrich Inc., St Louis, Missouri, USA) and activated mPEG-propionic acid *N*-hydroxysuccinimidyl ester (mPEG-SPA), mPEG MW 550 and ester MW 719 (Nektar Therapeutics, Huntsville, Alabama, USA), as previously described [59]. Briefly, BPEI and dry chloroform (Fisher Chemicals, Fairlawn, New Jersey, USA) were mixed together to dissolve the BPEI. A solution containing cholesteryl chloroformate and mPEG550-SPA in dry chloroform was added dropwise to the reaction mixture, with stirring followed by a 3–4-h incubation period. The mixture was then placed under vacuum to concentrate the solution and remove the residual chloroform. The resulting residue was dissolved in 1 mol/l aqueous HCl and stirred. This solution of PPC hydrochloride was again concentrated under vacuum, yielding a highly viscous material. The final PPC product was isolated by precipitation with acetone. The hygroscopic PPC hydrochloride was dried under vacuum and stored under argon at -20°C until use. Nuclear magnetic resonance was used to calculate the molar ratios of PEI to the functional groups CHOL and PEG. Target ratios were 1:2.5:1 (PEI:PEG:CHOL).

Polymer DNA complexes were generated by first preparing PPC and DNA stock solutions at the appropriate concentrations in lactose. Stock solutions of cationic polymer (5 mg/ml) and DNA (3 mg/ml) in water for injection were diluted in a lactose solution ranging from 0.3 to 3.0%: these were required to achieve a final 10% lactose concentration upon reconstitution. To generate the complexes, the DNA was then added to the PPC solution while stirring and was incubated for 15 min at room temperature. For the small-scale application in these studies, 500 μl of prepared formulation was added

to 2-ml borosilicate glass vials and placed into a freeze-dryer (FREEZONE freeze dry System; LABCONCO Corp., Kansas City, Missouri, USA). Vials were cooled to -34°C for 4 h before the start of the primary drying. After 24 h, the shelf temperature was raised to -20°C and kept under vacuum for another 24 h. Finally, the shelf temperature was raised to 4°C and vials were capped under vacuum. After the lyophilization process, dried cake was reconstituted with water to various concentrations ranging from 0.5 to 10 mg/ml for injection.

After the reconstitution of PPC/DNA, particle size and ζ -potential of the complex was measured by dynamic light scattering using a 90 Plus/BI-MAS Brookhaven particle size analyzer (Holtville, New York, USA). Specifically, 50 μl of formulation was added to 950 μl of milli-Q water in polystyrene cuvetts for analysis.

Biodegradable carmustine polymer fabrication

The BCNU-containing polymers were kindly provided by Dr H. Brem from the Department of Neurological Surgery, Johns Hopkins University School of Medicine, Baltimore, Maryland. These polymers were prepared as previously described [62]. Briefly, the poly(carboxyphenoxypropane-sebacic acid) polymers were obtained from Avi Domb (Hebrew University, Jerusalem, Israel) in 20:80 formulation. To refine the polymers before use, they were dissolved in dichloromethane (10% w/v), then immediately precipitated by adding them slowly to a stirred solution of diethyl ether and hexane (1:1 v/v). The precipitated polymer was then isolated by filtration, dried in a vacuum desiccator, packed under dry nitrogen, and stored in a freezer at -20°C until use. BCNU was incorporated into the refined polymers according to the method of Domb and Langer [63], to yield a polymer containing 10% BCNU by weight in 20:80 poly(carboxyphenoxypropane-sebacic acid) formulations. The BCNU-polymer mixtures were dissolved in dichloromethane to yield 10% solutions (w/v). The solvent was then evaporated in a vacuum desiccator. The resulting dried polymers were compression-molded into discs weighing approximately 10–12 mg using a stainless-steel mold with an internal diameter of 3.0 mm in a Carver Press (No. 400; Littlestown Hardware and Foundry Co., Littlestown, Pennsylvania, USA) at approximately 200 pound/inch². The individual weights of all the polymer discs were recorded.

Cells and cell culture

For in-vitro analysis of lyophilized formulations, COS-1 cells were plated in 12-well plates at 150 000 cell/well in 10% fetal bovine serum (FBS)-supplemented Dulbecco's modified Eagle's medium (DMEM) and grown to $\sim 80\%$ confluence. For transfection, a total of 4 μg of pmIL-12 was added to the cells (in the absence of FBS). To account for the differences in the DNA concentrations of the formulated material, an additional amount of FBS-

free DMEM was added to bring the total added volume to 500 μl , and the cells were incubated for 6 h 37°C . After incubation, the DMEM/formulation was aspirated from the wells and replaced with 1 ml of 10% FBS-supplemented DMEM and incubated for an additional 48 h. After transfection, supernatants were removed and analyzed directly for mIL-12 protein by enzyme-linked immunosorbent assay (R&D systems, Minneapolis, Minnesota, USA). The pmIL-12 construct contains two cytomegalovirus promoters driving each of the genes necessary for active IL-12 (p35 and p40), and has been described previously [64].

The murine glioma (GL261) was obtained from American Type Culture Collection (Rockville, Maryland, USA). The GL261 glioma is derived from a 3-methylcholanthrene-induced glioma syngeneic to C57BL/6 mice. The glioma cells were grown in DMEM supplemented with 10% FBS in the presence of streptomycin (100 $\mu\text{g}/\text{ml}$) and penicillin (100 U/ml). The cells were grown at 37°C in a humidified atmosphere with 5% CO_2 . Cultures were split every 3 days to ensure logarithmic growth.

Intracranial tumor establishment and treatments

Mice were anesthetized with an intraperitoneal injection of 0.1 ml of a stock solution containing ketamine hydrochloride 25 mg/ml, xylazine 2.5 mg/ml, and 14.25% ethyl alcohol diluted 1:3 in 0.9% NaCl. For stereotactic intracranial injections of tumor cells, the surgical site was shaved and prepared with 70% ethyl alcohol and Prepodyne solution (West Agro, Kansas City, Missouri, USA). After a midline incision, a 1-mm right parietal burr hole centered 2 mm posterior to the coronal suture and 2 mm lateral to the sagittal suture was made. Animals were then placed in a stereotactic frame and 1×10^5 GL261 tumor cells were delivered by a 26-gauge needle to a depth of 3 mm over a period of 3 min. The total volume of injected cells was 5 μl . The needle was removed, the site was irrigated with sterile 0.9% NaCl, and the skin was sutured with 4.0 nylon. Intracranial injection of pmIL-12/PPC was performed using the same technique 3 days after tumor inoculation. BCNU wafers were implanted 2 days after pmIL-12/PPC injection.

Histological and immunohistochemical evaluation

For toxicity studies and efficacy studies, animals were euthanized weekly and brains were subjected to histological inspection for signs of toxicity. Midtumor coronal cuts were subjected to histological (hematoxylin & eosin) staining and immunohistochemical analysis. Serial 10- μm paraffin sections of specimens were stained with rat antimouse monoclonal antibodies against CD4 (L3T4 clone #443043 at 1/25 dilution) or CD8 (Ly-2 clone #553829 at 1/50 dilution) (BD Bioscience; San Jose, California, USA). For mouse IL-12, rat antimouse IL-12 (p40/p70) LEAF monoclonal antibody, unconjugated,

clone C15.6 (BioLegend, San Diego, California, USA) was used. Primary antibodies were detected using the biotin–peroxidase system (BD Bioscience).

Statistical analysis

Survival was plotted using a Kaplan–Meier survival curve and statistical significance was determined by the Kruskal–Wallis nonparametric analysis of variance, followed by the nonparametric analog of the Newman–Keuls multiple comparison test. A *P* value of less than 0.05 was considered significant.

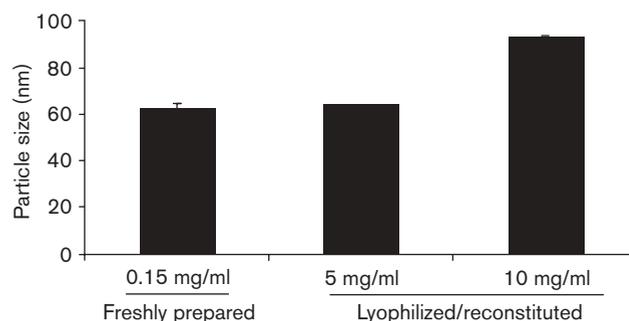
Results

Generation of highly concentrated murine plasmid encoding IL-12 (pmIL-12)/PPC

In this study, we generated highly concentrated pmIL-12/PPC, to administer a significant amount of intracranial injection at the lowest possible volume. Upon synthesis, pmIL-12/PPC was lyophilized and reconstituted at concentrations up to 10 mg/ml. To explore the possibility of aggregation secondary to the high concentration, particle size measurements of DNA/polymer were performed. The lyophilized PPC formulations were compared with unlyophilized freshly prepared PPC formulations. No complex aggregation of the reconstituted formulations was indicated, and particle sizes were all within acceptable ranges of < 250 nm (Fig. 1). The ζ -potentials of the complexes were as follows: 27.49 mV \pm 2.13 (0.15 mg/ml); 36.31 mV \pm 3.83 (5.0 mg/ml); and 31.94 mV \pm 0.83 (10 mg/ml). The results indicate that a strong positive ζ -potential of polymer/DNA complexes (> 20 mV) is preserved during the lyophilization and reconstitution process. These results are consistent with the retention of transfection activity of the corresponding formulations, demonstrating that the process of concentrating the transfection complexes does not significantly affect their surface properties or transfection activity. At all three DNA concentrations, the complexes were fully condensed and sufficiently positively charged (> 20 mV) for optimal charge interaction with the cell membrane; nevertheless, there were small differences in the absolute ζ -potential values between the formulations. We believe that these differences could be due to random interference in the gyrational rotation of the particles by PEG chains during the measurements.

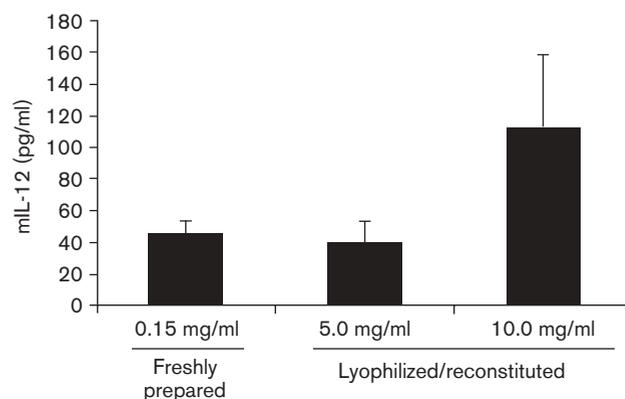
An in-vitro assay using COS-1 cells was used to confirm the functional properties of the lyophilized material. Transfection efficiencies of pmIL-12/PPC reconstituted at various concentrations were compared with that of pmIL-12/PPC, freshly prepared at 0.15 mg/ml (Fig. 2). No losses were observed in the transfection efficiency of the formulated plasmid that had been subjected to lyophilization. Of note, a two-fold increase in mL-12 expression was noted in cells transfected with mL-12/PPC complexes that had been reconstituted to 10 mg/ml.

Fig. 1



Physical properties of PPC. Particle size measurements of DNA/polymer complexes that have been formulated at 0.15 mg/ml (DNA concentration), lyophilized, and reconstituted at 5.0 or 10 mg/ml. The particle size of formulated prelyophilized material is also presented. Particle size was determined by dynamic light scattering. PPC, polyethylenimine covalently modified with methoxypolyethyleneglycol and cholesterol.

Fig. 2

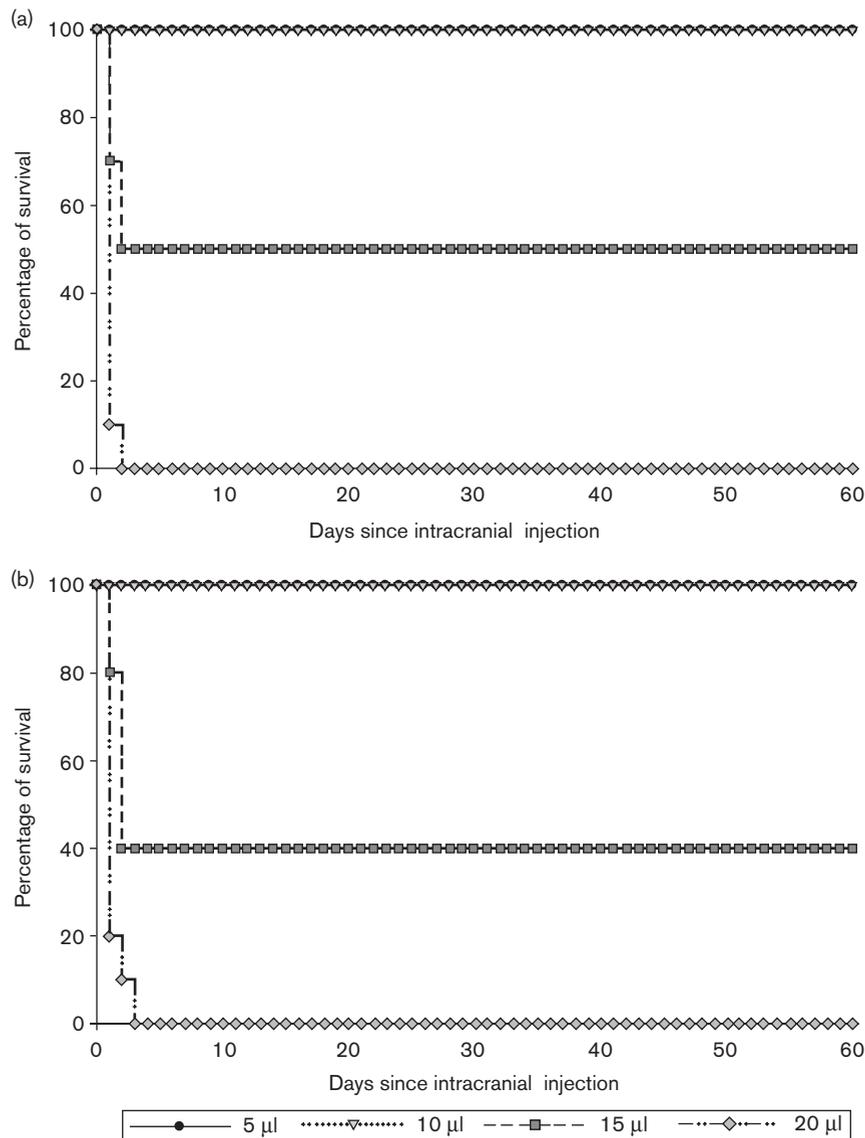


Transfection efficiency of lyophilized mL-12/PPC. Cos-1 cells were transfected with mL-12/PPC formulated to various concentrations. Comparisons were made between freshly prepared (nonlyophilized) mL-12/PPC (0.15 mg/ml) and mL-12/PPC that had been lyophilized and reconstituted at 5.0 or 10 mg/ml. For transfection comparison, the same total amount of DNA (4 μ g) was used for each group. Values are presented as means \pm SD. pmIL-12, murine plasmid encoding IL-12; PPC, polyethylenimine covalently modified with methoxypolyethyleneglycol and cholesterol.

Assessment of toxicity after intracranial injection of murine plasmid encoding IL-12 (pmIL-12)/PPC

To determine the potential toxicity associated with intracranial injection of pmIL-12/PPC, different volumes of this complex were injected intracranially at a concentration of 10 mg/ml, and animals were then followed up for survival (Fig. 3). The volumes injected were 5, 10, 15, and 20 μ l. Injections of up to 10 μ l of PPC or pmIL-12/PPC led to 100% survival. Injections of 15 μ l

Fig. 3

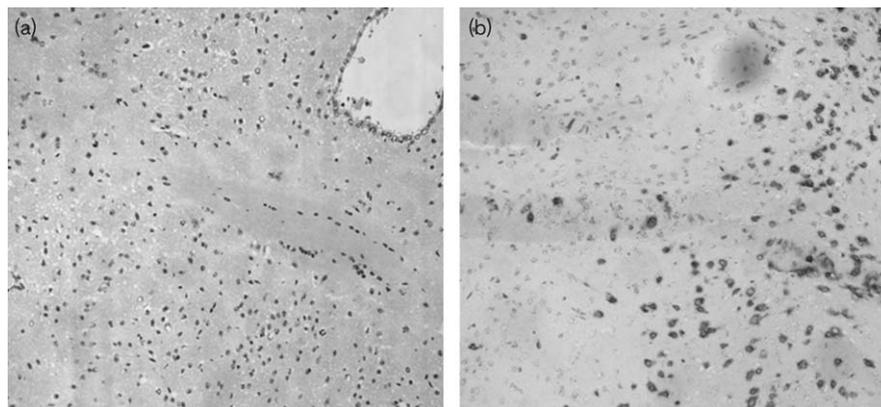


Kaplan–Meier survival graphs after intracranial injection of pmIL-12/PPC. Toxicity associated with the injection of increasing volumes of (a) empty PPC polymer and (b) polymer pmIL-12/PPC (at 0.5 mg/ml). Injection of normal saline (not shown) overlaps with findings of (a) and indicates that the toxicity is related to the volume of intracranial injection rather than to IL-12. Differences among groups were statistically significant ($P < 0.05$). pmIL-12, murine plasmid encoding IL-12; PPC, polyethylenimine covalently modified with methoxypolyethyleneglycol and cholesterol.

of PPC or pmIL-12/PPC led to 50 and 40% survival, respectively. An intracranial injection of 20 µl was associated with a lack of survival of all mice. These experiments show that volumes up to 10 µl of pmIL-12/PPC or PPC alone are safe for intracranial injection in mice, independent of the presence of pmIL-12. At a dose of 15 µl, a similar lethality was observed for empty PPC and pmIL-12/PPC. Although a tendency for a higher toxicity was observed for pmIL-12/PPC at this particular volume (10% difference when compared with empty PPC), we do not consider this finding significant as repeat experiments were equivocal. Injection of

normal saline (not shown) led to a similar survival as the one described for PPC injection (Fig. 3b). We thus attribute the toxicity observed to the volume of intracranial injection rather than to the PPC or pmIL-12/PPC. This conclusion is further supported by the histological analyses of the brain slices obtained on a weekly basis after treatment with 15–20 µl PPC or pmIL-12/PPC. We did not observe any evidence of hemorrhage, stroke, or tissue necrosis between the two groups. Of note, there was a significant infiltrate with CD4⁺ and CD8⁺ T-cells in the pmIL-12/PPC group.

Fig. 4



Histological examination of animal brains 1 month after intracranial injection of either an empty polymer (a) or pmIL-12/PPC (b) shows normal brain parenchyma and positive staining for IL-12 expression, respectively. pmIL-12, murine plasmid encoding IL-12; PPC, polyethylenimine covalently modified with methoxypolyethyleneglycol and cholesterol.

IL-12 expression in normal brain parenchyma after murine plasmid encoding IL-12 (pmIL-12)/PPC

To evaluate the induction of IL-12 expression driven by pmIL-12/PPC, immunohistochemical staining for IL-12 was performed on slices of brains from animals euthanized 14 days or 1 month after treatment. As a representative time point, data from mice euthanized 1 month after treatment is presented. Brain parenchyma of animals treated with PPC did not show any IL-12 staining (Fig. 4a); in contrast, brain parenchyma of mice injected with pmIL-12/PPC intracranially stained positive for IL-12 (Fig. 4b). This experiment demonstrates that the expression of IL-12 is specifically elicited by the pmIL-12/PPC complex. In addition, it can be concluded that the cytokine is present for at least 1 month after injection. Moreover, the presence of this cytokine in the brains of animals that remained alive until euthanized suggests that the actual expression of IL-12 does not cause lethal toxicity in brain. This is important to consider, as IL-12 expression outside the tumor margin is a possibility after intracranial administration of pmIL-12/PPC.

Therapeutic effect of murine plasmid encoding IL-12 (pmIL-12)/PPC in GL261 experimental murine gliomas

To assess the efficacy of pmIL-12/PPC, we studied the effect of pmIL-12/PPC treatment on the survival of mice bearing intracranial GL261 tumors. As BCNU is a chemotherapeutic agent currently available for the treatment of brain tumors [60,61], we also explored the effect of pmIL-12/PPC as an adjuvant therapy in addition to BCNU. Treatment with 3 μ l of pmIL-12/PPC at a concentration of 5.0 mg/ml led to no significant prolongation of survival when compared with mice bearing GL261 alone (median survival 39 vs. 33 days, respectively, $P > 0.05$). At this concentration, the addition of BCNU

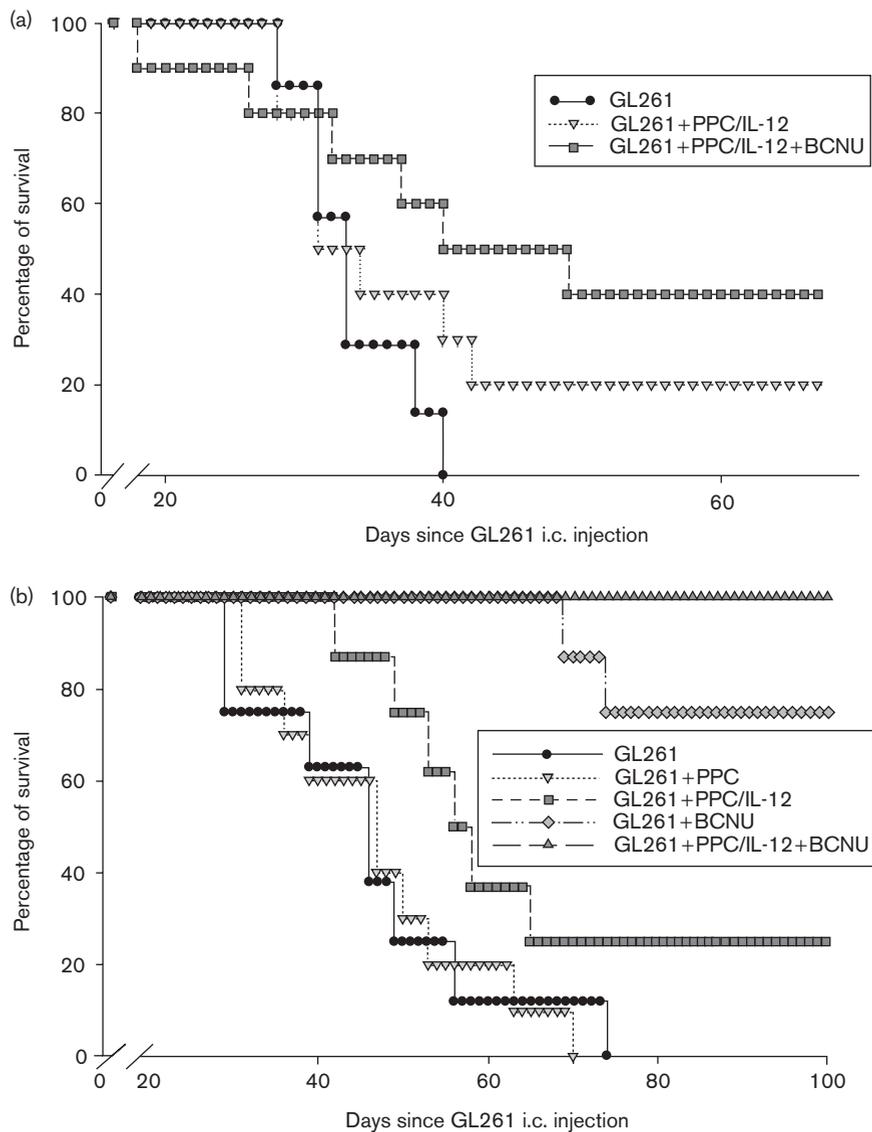
led to a significant increase in survival (median survival 47 days; 40% were long-term survivors; $P < 0.05$) (Fig. 5a). Injection of 3 μ l of pmIL-12/PPC at a concentration of 10 mg/ml led to considerable therapeutic effect with a significant increase in survival, compared with untreated mice (median survival 57 days; 25% long-term survival, 45 days for control; $P < 0.05$) (Fig. 5b). Treatment with BCNU led to a significant difference in survival when compared with untreated mice with 75% long-term survival (> 95 days; $P < 0.05$). At this higher concentration, the addition of BCNU to pmIL-12/PPC increased the survival to 100% for 95 days after treatment ($P < 0.0001$). These experiments suggest a significant therapeutic effect of pmIL-12/PPC in the treatment of experimental brain tumors. Furthermore, a synergistic effect is also demonstrated of BCNU chemotherapy and the antitumor properties of pmIL-12/PPC.

To explore the mechanism by which the pmIL-12/PPC injection led to an increase in survival, we examined T-lymphocyte infiltration in tumor tissue as an indication of antitumoral cellular immune response. To this end, histological examination of tumor samples was done 14 days or 1 month after treatment. Data from mice euthanized 1 month after treatment are presented. Immunohistochemical analysis of CD4 and CD8 showed positive infiltration of both cell populations in tumors treated with pmIL-12/PPC or pmIL-12/PPC + BCNU when compared with untreated tumors (Fig. 6).

Discussion

In this study, we examined the feasibility of delivering a transgene into experimental brain tumors with the aid of a polymer-based vehicle. First, concentrated

Fig. 5

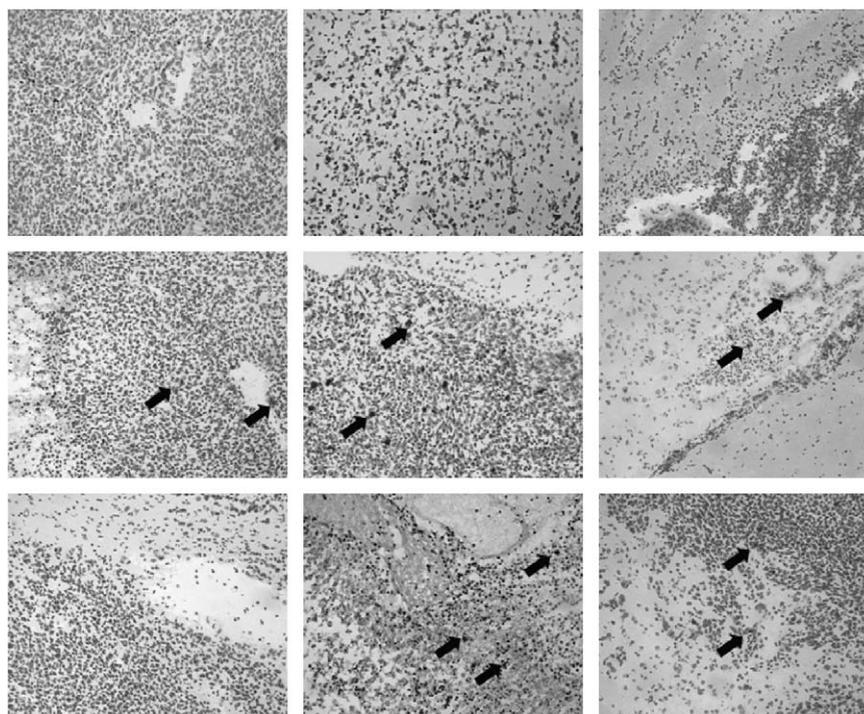


Kaplan-Meier survival graphs show the efficacy of pmIL-12/PPC with BCNU. First, for the pilot survival experiment (a), a volume of 3 µl of pmIL-12/PPC at 5 mg/ml concentration was injected. For the next survival experiment, a volume of 3 µl of pmIL-12/PPC at a concentration of 10 mg/ml was injected (b). BCNU, biodegradable carmustine; pmIL-12, murine plasmid encoding IL-12; PPC, polyethylenimine covalently modified with methoxypolyethyleneglycol and cholesterol; i.c., intracranial.

pmIL-12/PPC proved to be compatible with transgene expression in murine brains. pmIL-12/PPC and the resultant protein seem to be well tolerated by this route up to a final volume of 10 µl. This finding is relevant owing to the fact that despite initial aggressive testing in the clinic, recombinant IL-12 therapy for cancer has not advanced to the status of an approved therapy primarily owing to toxicity concerns. Systemic toxicity varies and includes influenza-like symptoms, fever or chills, nausea or vomiting, orthostatic hypotension, anorexia, diaphoresis, asthenia, headache, injection site reaction, pain,

myalgia, arthralgia, elevated aspartate and alanine aminotransferase levels, and anemia or exacerbation of hemolytic anemia: the latter possibly led to the death of one patient who had leukemia [38,39,41]. In this study, we hypothesized that local intracranial delivery of an otherwise highly toxic therapeutic agent might be better tolerated and show increased efficacy vs. systemic delivery. Indeed, local delivery of BCNU bypasses some of the systemic side effects of BCNU, and has been approved by the FDA as a treatment for glioblastoma multiforme [61,65].

Fig. 6



Histological examination of GL261 brain tumors of mice euthanized 1 month after treatment. Tissue was stained with hematoxylin & eosin, or immunohistochemistry for CD4⁺ and CD8⁺ (arrows). Representative photomicrographs show CD4⁺ and CD8⁺ cell infiltration in groups of mice treated with pmIL-12/PPC, independent of the addition of BCNU to the treatment. BCNU, biodegradable carmustine; pmIL-12, murine plasmid encoding IL-12; PPC, polyethylenimine covalently modified with methoxypolyethyleneglycol and cholesterol.

We have shown that IL-12 can be produced in sufficient quantity to allow a significant therapeutic effect after intracranial injection. Treatment with pmIL-12/PPC led to a significant increase in survival compared with untreated mice: 25% long-term survivals (> 95 days). This survival is comparable with the one described by DiMeco *et al.* [48], a study in which irradiated 9L cells that produced IL-12 were shown to be effective in the treatment of intracranial gliomas. In that study, all animals injected with only wild-type 9L cells (control group) died, with a median survival time of 25 days (range 23–37 days). Rats injected with a mixture of 9Lwt and 9L-IL12 cells (simultaneous treatment) had a significantly prolonged survival (median 53.5 days; range 25–112 days; $P = 0.0003$), with 25% being long-term survivors. Rats bearing established tumors, which were subsequently treated with an intratumoral injection of 9L-IL-12 cells (delayed treatment), also had significant prolongation of survival time (median 51.1 days; range 23–112 days; $P = 0.0018$) compared with controls, with 20% being long-term survivors. In this study, treatment with BCNU led to a significant difference in survival compared with untreated mice: 75% long-term survivals (> 95 days). Most importantly, the combination

of BCNU and pmIL-12/PPC led to a survival of 100% for 95 days after treatment.

This therapeutic outcome might be related to the prolonged expression of IL-12 after its administration as a transgene. It has been suggested that for an optimal antitumoral effect, cytokines must be secreted over an extended period of time. For instance, in a previous study in which IL-12 was shown to inhibit tumor establishment at a distant site in a murine renal carcinoma model, a single systemic administration of recombinant IL-12 had a relatively weaker effect than that of local IL-12 [66]. The difference in the therapeutic effect was attributed to the persistent and local expression of IL-12 protein. Specifically, IL-12 expression as a result of a single local injection of IL-12 cDNA lasted for at least 15 days. In contrast, systemic IL-12 administration led to a half-life of less than 24 h. Gene therapy with pmIL-12/PPC might thus offer a significant advantage over conventional recombinant protein therapy, in that it can provide sustained concentrations of IL-12 within the tumor environment, sufficient to be therapeutic.

The mechanism by which IL-12 elicits an effective antitumoral response has been previously described in the setting of gliomas and other cancers [35–37,45,46]. Briefly, IL-12 promotes the activation and infiltration by cytotoxic lymphocytes in tumor tissue. This appears to be mediated via the presence of CD8⁺ cytotoxic T-cells [67–69]. In this study, CD8⁺ cell infiltration was documented in mice that had been treated with pmIL-12/PPC. We attribute part of the antitumoral effect of IL-12 gene therapy to the promotion of a cytotoxic immune response. Additionally, although not studied in this case, the contribution of the antiangiogenic feature of IL-12 remains a likely possibility. In a peritoneal disseminated mouse model of ovarian cancer, intraperitoneal administration of pmIL-12/PPC enhances IFN- γ levels, inhibits vascular endothelial growth factor levels in ascites fluid, and enhances animal survival (J. Fewell, personal communication).

The combination of IL-12 and gene therapy, so-called immunogene therapy, shows promise in the setting of brain tumors. The PPC polymer, which through lyophilization allows for formulation at a high DNA concentration that is not possible with other polymeric formulations, seems to be an efficient delivery vehicle for this purpose. At the same time, a combined treatment of IL-12 and local chemotherapy clearly works in synergy to extend the survival of animals with experimental brain tumors. This strategy suggests a role for this treatment as an adjuvant to the current therapeutic tools available for malignant gliomas. Further assessment of this therapy is needed, as it can eventually lead to a benefit in the clinical setting.

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