Injectable Chemotherapeutic Microspheres and Glioma II: Enhanced Survival Following Implantation into Deep Inoperable Tumors

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Purpose. Delivery of chemotherapeutics using implantable, biodegradable polymers provides a potentially powerful method of treating brain tumors. The present studies examined the ability of injectable microspheres, formulated to release carboplatin or BCNU for 2–3 weeks, to enhance survival in a rodent model of deep, inoperable glioma.

Methods. Rat glioma (RG2) cells were implanted into the striatum of rats. In a first experiment, the tumors were allowed to grow for 3 days, followed by either no treatment, bolus chemotherapy (100 μg), or implantation of microspheres containing 10, 50, or 100 μg of carboplatin. The microspheres were implanted, via hypodermic injection, directly into the center of the small, 3-day-old tumors. In a second experiment, tumors grew for 8 days prior to treatment with either carboplatin- or BCNU-loaded microspheres. The microspheres were then injected either directly into the center of these larger tumors or into three sites along the perimeter of the tumor. Separate sets of animals received bolus chemotherapy (100 μg) into either the tumor center or around the tumor perimeter.

Results. Injection of carboplatin-loaded microspheres into the center of the small 3 day old, tumors produced dose-related increases in survival. When injections of carboplatin- or BCNU-loaded microspheres were made into the center of the larger, 8-day-old tumors, survival was not enhanced. However, when the microspheres were injected along the perimeter of the larger tumors, sustained-release chemotherapy did significantly prolong survival. Bolus chemotherapy was less effective than sustained release chemotherapy.

Conclusions. Together, these data: (1) demonstrate that sustained delivery of chemotherapy in or near the tumor site is superior to equipotent bolus doses in inoperable tumors, (2) demonstrate that injection of sustained release microspheres into the tissue surrounding a growing tumor may provide superior effects over injections directly into the tumor mass, and (3) suggest that this approach may provide a useful means of selectively delivering chemotherapeutics to tumors or portions of tumors that cannot otherwise be treated with conventional surgical approaches.

KEY WORDS: glioma; sustained release; microsphere; carboplatin; BCNU.

INTRODUCTION

Malignant gliomas are commonly treated with a combination of surgery, radiation therapy, and systemic chemotherapy. Despite aggressive treatment regimens, the median survival of patients remains approximately one year from the time of diagnosis and cases of long-term disease-free survival in adults are rare (1). A significant limiting factor in treating glioma is the inability to deliver therapeutic concentrations of chemotherapeutic drugs to the tumor without incurring unacceptable systemic side effects. Developing approaches to increase local exposure of brain tumors to chemotherapeutic drugs, without increasing systemic toxicity, would be a valuable means of optimizing the antitumor activity of currently used chemotherapeutic drugs.

Implantable, biodegradable polymers provide a useful and practical means of maximizing the efficacy of antineoplastic drugs by providing vehicles for local and sustained drug delivery directly to the tumor. Polymeric carriers for chemotherapeutic agents have been extensively evaluated in animal models of brain tumors (2–6) and most recently in the treatment of human glioma (10–12). The most noteworthy effort to date uses poly[bis(p-carboxyphemoxy)]propane-sebacic acid (PCPP-SA) copolymer disks that release 1, 3-bis[2-chloroethyl]-1-nitrourea (BCNU). Following an extensive series of preclinical (13 for a review) and clinical studies (7–10), FDA approval was recently granted for the use of BCNU-loaded polymer disks as an adjunctive treatment to resection of glioma. Following surgical resection of tumors, the polymer disks are placed into the resulting cavity where the BCNU is released to diffuse into the surrounding tissue and residual tumor mass. Using this approach, statistically significant patient benefit has been observed with median survival increased by 8 weeks (10).

Polymer devices such as disks can only be used in situations where the tumor is surgically accessible to create a cavity. They cannot be applied when tumors are located in surgically inaccessible portions of the brain, or are too numerous. Without the ability to surgically remove these tumors or deliver therapeutic levels of chemotherapy to them, the tumor grows and the patient inevitably dies. If adequate concentrations of antineoplastic drugs could be delivered directly to these normally untreatable tumors, greater patient benefit might be observed. One means of permitting delivery directly to the site of normally inoperable tumors is the use of polymeric microspheres. Microspheres can be formulated to provide excellent in vivo release kinetics, delivering high local concentrations of drugs for predefined periods of time ranging from days to months. Microspheres have been proven to be efficient systems for delivery of a wide range of chemotherapeutic drugs (12–14) and can be easily injected as a suspension allowing drug delivery into virtually any site of the brain with minimal invasiveness (15,16).

Using an animal model of surgically resected glioma, we previously reported the first direct evidence that injections of sustained release microspheres into the tissue surrounding the tumor cavity provide superior survival effects over that obtained with injections into the cavity (17). Injections of sustained release microspheres into the tissue surrounding the resection cavity were intended to overcome the limited diffusion of drugs within brain tissue, allowing the tumor to be treated both locally and in regions of likely tumor infiltration. This approach might also represent a significant advance in the ability to treat inoperable tumors, since the microspheres could easily be injected into either the tumor itself and/or into the tissue surrounding

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ABBREVIATIONS: BCNU, 1, 3-bis[2-chloroethyl]-l-nitrourea; PLG, poly (L-lactide) co-glycolide); RG2, rat glioma; PVA, polyvinyl acetate; PBS, phosphate-buffered saline; i.v., intravenous.

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the tumor. The following experiments are the first to evaluate the potential value of sustained release chemotherapy into the tissue surrounding a growing inoperable tumor mass versus into the tumor itself. Direct comparisons are made between sustained release formulations within the tumor and into the tumor perimeter, relative to equipotent bolus injections into both sites. The efficacy produced by two different chemotherapeutic drugs, carboplatin and BCNU, was tested in this model by injecting the microspheres either directly into the tumor or into the tissue along the perimeter of the tumor, and monitoring the animals for survival. The results extend prior observations following surgical resection of glioma (17), while providing the first evidence for the superiority of peritumoral injections over direct injections into a deep inoperable tumor as a means of interstitial chemotherapy. If the superiority of peritumoral over direct tumoral chemotherapy is confirmed in human glioma trials, the future use of polymeric delivery systems for treating brain tumors could be dramatically altered.

MATERIALS AND METHODS

Subjects

Male Fischer rats (N = 341; 200–220 g; Taconic Farms, Germantown, NY) were used in the following studies. The rats were housed in pairs in polypropylene cages with free access to food and water. The vivarium was maintained on a 12 h light: 12 h dark cycle with a room temperature of 22 ± 1°C and relative humidity level of 50 ± 5%. All studies were in compliance with the rules set forth in the Guide for the Care and Use of Laboratory Animals.

Tumor Cell Implantation

RG2 cells were maintained and prepared for implantation as previously described (18). Rats were anesthetized using an intramuscular injection of a solution containing ketamine (33 mg/ml), xylazine (10 mg/ml) and acepromazine (1.6 mg/ml) and placed in a stereotaxic instrument. Using a 10 μl Hamilton syringe with a 22 gauge needle, RG2 cells were injected unilaterally into the striatum (1×10^5 cells/5 μl) at the following coordinates; A-P (+2.0 mm), L (+3.0 mm) and V (−6.5 mm) (19).

Fabrication of Carboplatin- and BCNU-Loaded Microspheres

PLG microspheres were fabricated for sustained release of carboplatin and BCNU as previosuly described (17). Carboplatin-loaded (Sigma Chemical) microspheres (PLG, Microsorb 50/50 DL, MW = 10 kD, Alkermes Inc., Wilmington, Ohio) were fabricated by a coacervation process with a carboplatin loading density of 10% (w/w). BCNU-loaded (Sigma Chemical) microspheres were fabricated by a solvent evaporation process with a loading density of 15% (w/w).

Survival Following Implantation of Carboplatin-Loaded Microspheres

An initial study characterized the survival benefit produced by sustained delivery of carboplatin in animals bearing small, 3 day old tumors. RG2 cells were implanted unilaterally into the striatum and 3 days later, the same animals received injections of microspheres containing carboplatin, or a bolus injection of carboplatin, directly into the center of the tumor at the same coordinates used to implant the RG2 cells. For implantation, the microspheres were suspended (10% PLG w/v) in a solution of 0.9% saline, 0.1% Tween and 3.0% carboxymethylcellulose (low viscosity). Identical amounts of microspheres were injected in all cases by adding blank microspheres to the suspension. Microspheres (1 mg/10 ul) were stereotaxically injected at a rate of 2 ul/minute using a 10 ul Hamilton syringe with an attached 23 gauge needle. Animals were assigned to one of 5 treatment groups: (1) no treatment (n = 15), (2) a bolus injection of 100 μg of carboplatin, (n = 12), (3) 10 μg sustained release carboplatin (n = 10), (4) 50 μg sustained release carboplatin (n = 13), or (5) 100 μg sustained release carboplatin (n = 16).

A second series of experiments examined the effects of sustained release carboplatin on larger, 8 day old striatal tumors. Eight days following tumor implantation, rats were assigned to one of 4 treatment groups: (1) 100 μg carboplatin as a bolus (n = 10), (2) 10 μg sustained release carboplatin (n = 12), (3) 50 μg sustained release carboplatin (n = 13), or (4) 100 μg sustained release carboplatin (n = 10). All injections were made directly into the center of the tumor at the same coordinates used for implantation of the RG2 cells.

These experiments also directly compared the survival produced by sustained release following implantation of microspheres directly into the center of the tumor vs the tissue along the perimeter of the tumor. For implantation of the microspheres into the tissue along the perimeter of the tumor, animals received the same total amount of sustained release carboplatin that was delivered directly into the tumor, except that it was equally divided into 3 separate 3.3 ul aliquots. Eight days following tumor implantation animals were assigned to one of 4 treatment groups: (1) 100 μg carboplatin (33.3 μg/site) as a bolus (n = 19), (2) 10 μg (3.3 μg/site) sustained release carboplatin (n = 20), (3) 50 μg (16.7 μg/site) sustained release carboplatin (n = 22), or (4) 100 μg (33.3 μg/site) sustained release carboplatin (n = 22). Implants were made into 3 sites at the following coordinates along the perimeter of the tumor: A-P (+2.85 mm), L (+3.0 mm) and V (−6.5 mm); A-P (+1.15 mm), L (+2.0 mm) and V (−6.5 mm); A-P (+1.15 mm), L (+4.0 mm) and V (−6.5 mm). These coordinates were derived from prior studies in glioma and were calculated to place the microspheres approximately 0.5 mm outside of the tumor perimeter (20).

Survival Following Implantation of BCNU-Loaded Microspheres

A second series of experiments examined the effects of sustained release BCNU in animals bearing larger, 8 day old striatal tumors. Eight days following tumor implantation, rats were assigned to one of 5 treatment groups. For direct injections into the center of the tumor, animals received either (1) no treatment (n = 15), (2) a bolus injection of BCNU (n = 15), (3) 10 μg sustained release BCNU (n = 15), (4) 50 μg sustained release BCNU (n = 15), or (5) 100 μg sustained release BCNU (n = 15). Again, all injections were made directly into the center of the tumor at the same coordinates used for implantation of the RG2 cells.

The survival produced by sustained release of BCNU following implantation of microspheres directly into the center of the tumor vs the tissue along the perimeter of the tumor was directly compared eight days following tumor implantation. For implantation into the tissue along the tumor perimeter, animals were assigned to one of 4 treatment groups: (1) 100 μg BCNU (33.3 μg/site) as a bolus (n = 15), (2) 10 μg (3.3 μg/site) sustained release BCNU (n = 15), (3) 50 μg (16.7 μg/site) sustained release BCNU (n = 15), or (4) 100 μg (33.3 μg/site) sustained release BCNU (n = 15). Following treatment in all survival studies, animals were monitored daily for signs of ill health and any animal showing signs of morbidity was euthanized via CO_2 asphyxiation and that date recorded for calculating survival data.

Statistical Analysis

Survival after each treatment was analyzed using Kaplan-Meier survival curves. Non-parametric Kruskal-Wallis statistics were used to determine overall treatment effects using the day of death as the nonparametric variable (JMP, SAS Institute Inc., Cary, N.C.). The nonparametric modification of the Neuman-Keuls test was used for subsequent pair-wise comparisons. Minimal statistical significance in all cases was defined as $p < 0.05$.

RESULTS

Survival Following Implantation of Carboplatin-Loaded Microspheres

Quantitation of H&E sections from a separate series of animals determined that the maximal cross-sectional tumor area of the 8 day old tumors (N = 6) was 4.39 ± 0.51 mm^2 for the 8 day old tumors and 1.26 ± 0.76 mm^2 for the 3 day old tumors (N = 6). The striatal RG2 tumor was uniformly fatal to all non-treated animals with a median survival of 18 days and all animals dying by 22 days post-tumor implantation (Fig. 1). Direct injections of carboplatin-loaded microspheres into the center of the tumor produced significant, dose-related increases in survival. Sustained release of 10 μg of carboplatin did not impact survival ($p > 0.10$). Conversely, 50 μg of sustained release carboplatin significantly increased median and maximum survival by 155% and 150% respectively, relative to no treatment ($p < 0.001$). 100 μg of sustained release carboplatin was the most effective dose tested and increased median and maximum survival by 178% and 227%, relative to no treatment ($p < 0.001$). A bolus injection of carboplatin (100 μg) modestly increased median survival by 33% and maximum survival by 36%, relative to no treatment ($p < 0.05$).

While direct injections of microspheres into the center of the tumor significantly prolonged survival, these effects were achieved in animals bearing relatively small (three day old) tumors. To further examine the potential of localized, sustained release therapy, we examined the effects of microspheres injected both intra- and peritumorally on survival in animals bearing larger, 8 day old tumors (Fig. 2). Direct injections of microspheres into the center of the 8-day tumor were much less effective than identical injections into the center of the smaller 3 day old tumors. Sustained release of carboplatin produced a modest increase in survival at the largest dose (100 μg) tested with median and maximum survival increased by 39% and 23%, respectively ($p < 0.01$). In contrast, injections of carboplatin-loaded microspheres into the tissue along the perimeter of the tumor significantly enhanced survival (Fig. 3). The effect was dose-related, with the lowest dose of carboplatin (10 μg) having no effect ($p > 0.10$), 50 μg increasing median survival 122% and maximum survival 123% ($p < 0.001$) and 100 μg of carboplatin increasing median survival by 189% and maximum survival by 191% ($p < 0.001$). A bolus injection of

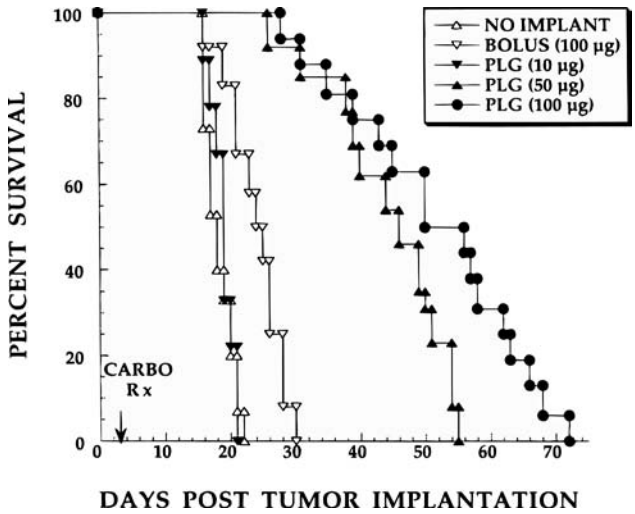

Fig. 1. Survival of rats bearing small, 3-day-old gliomas in striatum following a bolus injection of carboplatin (100 μg) or implantation of carboplatin-loaded microspheres (10 μg, 50 μg, or 100 μg total carboplatin) directly into the tumor center. While a bolus injection of carboplatin modestly prolonged survival, sustained release of carboplatin produced significant dose-related increases in survival relative to animals receiving no treatment.

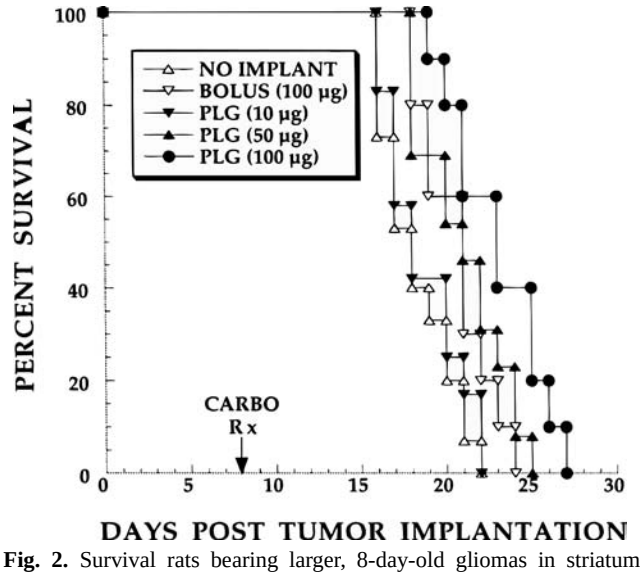

Fig. 2. Survival rats bearing larger, 8-day-old gliomas in striatum following a bolus injection of carboplatin (100 μg) or implantation of carboplatin-loaded microspheres of (10 μg, 50 μg, or 100 μg total carboplatin) directly into the center of the tumor. Only modest effects were achieved with sustained release directly into the center of these larger 8-day-old tumors, relative to the robust effects achieved with sustained release into the smaller 3-day-old tumors (Fig. 1). Bolus injections of carboplatin did not produce statistically significant increases in survival.

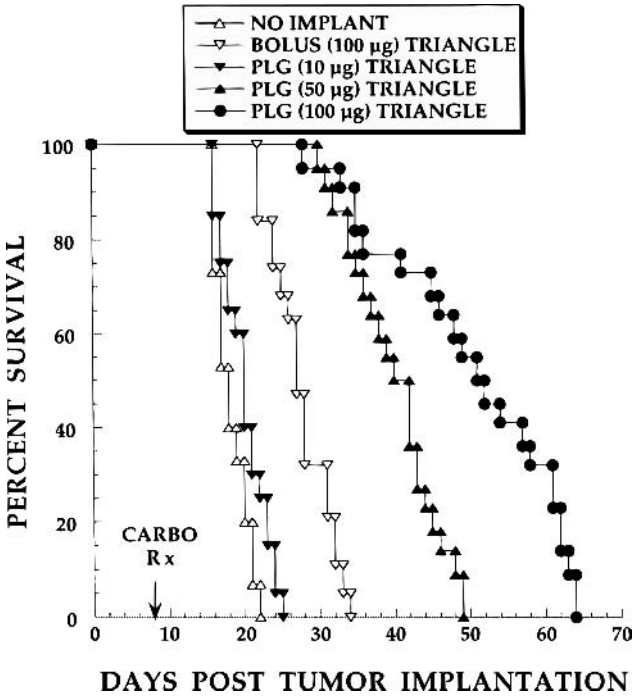

Fig. 3. Survival following a bolus injection of carboplatin (33.3 μg/site) or implantation of carboplatin-loaded microspheres (3.3 μg, 16.67 μg, or 33.3 μg/site) into 3 sites around the perimeter of an 8-day-old striatal glioma. The same total amount of carboplatin injected directly into the tumor was equally dispersed over the 3 implant sites (i.e., 33.3 μg/site for a total of 100 μg). Sustained release of carboplatin produced significant, dose-related increases in survival relative to animals receiving no treatment as well as relative to the modest effects achieved with injections directly into the tumor (Fig. 2). Bolus chemotherapy also prolonged survival, although these effects were significantly less than those acheived with sustained release.

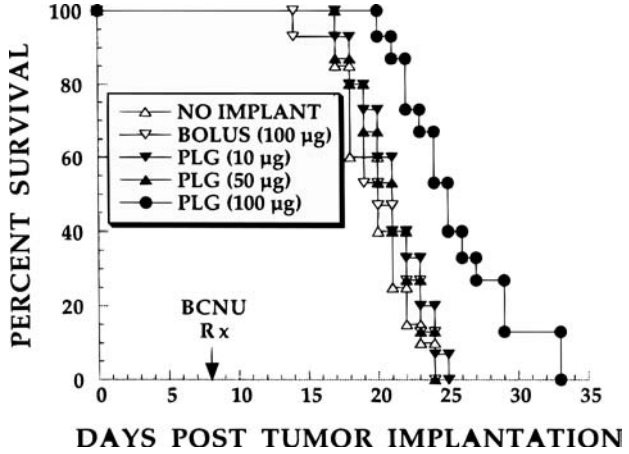

Fig. 4. Survival following a bolus injection of BCNU (100 μg) or implantation of BCNU-loaded microspheres (10 μg, 50 μg, or 100 μg total BCNN) directly into the center of an 8-day-old striatal glioma. Only the highest dose of sustained release BCNU (100 μg) prolonged survival and these effects were modest compared to the effects achieved with sustained release of BCNU along the perimeter of the tumor (Fig. 5). Bolus injections of BCNU did not prolong survival.

carboplatin (100 μg) along the perimeter of the tumor (100 μg) also improved median survival by 44% and maximum survival by 55% ($p < 0.01$), though this effect was significantly less than that achieved with sustained release ($p < 0.001$).

Survival Following Implantation of BCNU-Loaded Microspheres

Injections of BCNU-containing microspheres directly into the center of 8 day old striatal tumors versus the perimeter of the tumor produced similar effects to those observed with sustained release of carboplatin. When the BCNU-loaded microspheres were injected directly into the center of the tumor, only the highest dose (100 μg) significantly prolonged survival, as evidenced by a 25% increase in median survival and a 38% increase in maximum survival ($p < 0.01$). A bolus injection of BCNU (100 μg) did not impact survival ($p > 0.10$) (Fig. 4).

As was seen with carboplatin, injections of BCNU-loaded microspheres injected into the perimeter of the tumor produced robust increases in survival (Fig. 5). The effect was dose-related, with the lowest dose of carboplatin (10 μg) having no effect, 50 μg increasing median survival 55% and maximum survival 100% ($p < 0.001$) and 100 μg of carboplatin increasing median survival by 105% and maximum survival by 129% ($p < 0.001$). A bolus injection of carboplatin (100 μg) along the perimeter of the tumor (100 μg) improved median survival by 25% and maximum survival by 71% ($p < 0.01$), though this effect was less than that achieved with sustained release ($p < 0.001$). In general, the survival effect of sustained release BCNU was

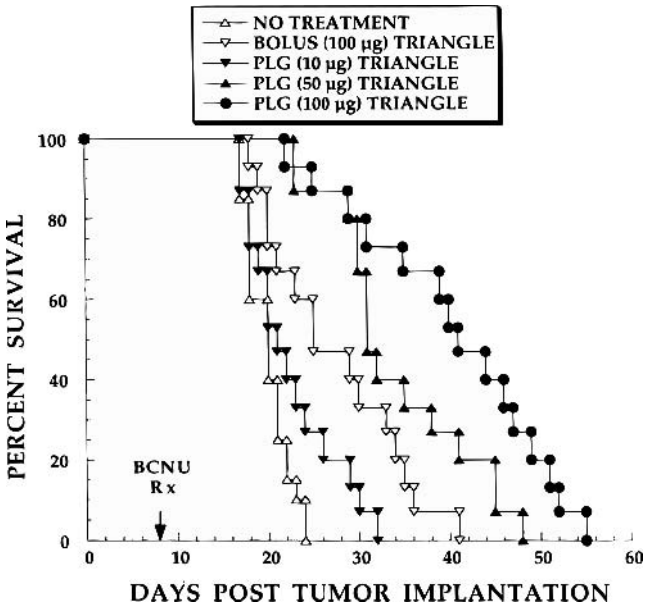

Fig. 5. Survival following a bolus injection of BCNU (33.3 μg/site) or implantation of BCNU-loaded microspheres of (3.3 μg, 16.67 μg, or 33.3 μg/site) into 3 sites within the tissue along the perimeter of an 8-day-old striatal glioma. The same total amount of BCNU injected directly into the tumor was equally dispersed over 3 implant sites (i.e., 33.3 μg/site for a total of 100 μg). Sustained release of BCNU into the perimeter of the tumor BCNU prolonged survival in a dose-dependent manner. Bolus chemotherapy also prolonged survival, although these effects were significantly less than those achieved with sustained release. Note also that the beneficial effects of BCNU were slightly less than those obtained with carboplatin (Fig. 4).

less than that achieved with sustained release carboplatin. For example, sustained release of 50 μg of carboplatin increased median survival as much as 100 μg of sustained release BCNU.

DISCUSSION

The results presented here establish several fundamental points regarding the use of sustained release microspheres as a treatment for normally inoperable brain tumors. They establish that: (1) microspheres can be easily injected into the brain to provide sustained release of chemotherapeutic drugs into a deep inoperable tumor bed as well as the tissue along the perimeter of that tumor, (2) sustained delivery of chemotherapy is superior to equipotent bolus doses, and (3) injections of sustained release microspheres into the tissue surrounding a growing inoperable tumor mass may be more effective than injections directly into the tumor itself.

Sustained release of carboplatin and BCNU was superior to bolus injections, even though equivalent total amounts of drug were administered in the two methods. While the bolus injections produce higher initial drug concentrations within the tumor, the increases in drug levels are short-lived compared to that achieved with sustained release. In recent studies characterizing these same microspheres, atomic absorption spectrophotometry confirmed that tissue levels of platinum remained elevated for two weeks following sustained release, in contrast to a 99% reduction in levels 3 days after a bolus injection of carboplatin (17). In both those studies (20) and the present studies, sustained release, but not bolus administration, of carboplatin significantly prolonged survival. The superior effects of sustained release over bolus injections in glioma-bearing rats has been reported in other studies (17,21) and clearly points out the benefits of maintaining locally elevated levels of chemotherapeutic within the tumor.

Systemic chemotherapy is ineffective, in large part because the concentrations of chemotherapeutic drugs within the tumor and surrounding tissue are not high enough to kill tumor cells. Interstitial chemotherapy using injectable microspheres elevates drug concentrations locally within the tumor for a prolonged period of time. Prior studies characterizing the microspheres used in the present studies revealed that carboplatin is released over a 3 week period, during which time tissue levels of platinum remain elevated (17). The higher levels of chemotherapeutic both reaching and remaining within the peritumoral region at therapeutic levels for prolonged periods of time enhances survival over that achieved with systemic chemotherapy, even when the systemic doses are orders of magnitude greater. For example, systemic (intravenous) carboplatin (10 mg/kg) given on days 7 and 9 following implantation of RG2 cells into the striatum of rats (e.g., identical model to that employed in the current studies) enhanced median survival by 52% relative to animals receiving no treatment (20). In contrast, sustained delivery of carboplatin increased median survival by 189% when the microspheres were injected along the perimeter of the tumor. The benefits of sustained release occurred even though the animals treated with systemic carboplatin received approximately 50 times the highest total dose delivered via sustained release.

The beneficial effects of sustained release were dependent on both the size of the growing tumor mass as well as the location of the microsphere injections. Injections of carboplatin-loaded microspheres into the center of the smaller 3 day old tumors significantly prolonged survival. However, when the tumors were allowed to grow for 8 days prior to injecting the microspheres, the same injections were ineffective. Numerous prior experiments have demonstrated the limited spatial distribution and steep concentration gradients of numerous compounds injected directly into the brain (22–24). We recently reported that carboplatin diffusion from microspheres is restricted primarily to the brain tissue within 0.5 mm of the injection site (17). Even though diffusion of carboplatin from the microspheres is limited, the small size of the 3 day old tumors likely allows the majority of the tumor to be exposed to cytotoxic drug concentrations high enough to prolong survival. In contrast, as the tumor grows larger, the diffusion distance required to reach the outer, rapidly expanding, portions of the tumor is likely too great to be reached by therapeutic concentrations of carboplatin. The importance of overcoming the restrictions imposed by diffusion to reach the outer, more rapidly growing portions of the tumor is further highlighted by the robust survival effects achieved when the microspheres were injected into the tissue along the perimeter of the larger 8 day old tumors.

While passive diffusion of injected agents is limited in brain tissue, additional mechanisms of fluids movement exist within the brain that are capable of further enhancing the distribution of drugs. An organized flow of fluids exists within the brain creating pressure gradients that push extracellular fluids by bulk flow along white matter tracts and perivascular spaces (25,26). These convection currents create paths of least resistence that provide a means for drugs injected into the brain to disperse to more distal sites than could be predicted from simple diffusion. Interestingly, these same pathways of low resistence provide a similar opportunity for tumor cells to migrate and clinical studies have indeed verified that white matter tracts are a common site of tumor recurrence (28–30). The presence of fluid convection systems within the brain also has important implications for the use of interstitial chemotherapy via microsphere injections. Knowledge of the organization of this system might provide important information about the dissemination of tumors outside the conventional surgical or radiotherapy field. With this knowledge, it might be easier to anticipate the areas most likely for tumor recurrence and, in turn, the most beneficial sites of microsphere injection. The ability to easily inject microspheres into the tissue within and surrounding brain tumors increases the likelihood of delivering adequate drug concentrations to tumor cells as they migrate from the primary tumor mass. Moreover, multiple injections of microspheres could be made that intentionally target those regions of higher bulk flow in an attempt to minimize successful tumor cell migration. The ability to sculpt a drug field that encompasses the tumor, the peritumoral region and nearby areas of probable tumor cell migration, maximizes the opportunity to treat both the primary tumor site and its most likely route of infiltration.

In summary, the data from these studies detail several new and compelling findings regarding the use of sustained release chemotherapy for treating inoperable glioma. Using an animal model of deep, inoperable glioma, the superiority of sustained release over bolus drug injections was confirmed. Furthermore, the results provide the first direct evidence that injections of

sustained release microspheres into the tissue surrounding a growing tumor mass may provide superior effects over injections into the tumor itself. Given the typical constrained drug diffusion within the brain, the use of injectable microspheres implanted into multiple peritumoral sites might provide one means of improving the limited spatial drug diffusion from the implantation site. Further work is required to establish the generality of the effects observed, especially in human gliomas. If confirmed in human glioma studies, those data would suggest that patients might benefit significantly from injection of microspheres that provide a localized and sustained delivery of chemotherapeutic drugs directly to region that are normally inacessible to surgical intervention.

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