Novel Antitumor Effect of Carboplatin Delivered by Intracerebral Microinfusion in a Rat Malignant Glioma Model

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Abstract
Carboplatin loaded osmotic mini-pumps were implanted in 24 9L malignant glioma-bearing rats to investigate the implications of direct intracerebral microinfusion. Carboplatin using 0.1 mg/ml (low dose group) or 1.0 mg/ml (high dose group) with eight rats in each group, or 5% D-glucose (control group) in eight rats were infused at 1 μl/hr for 7 days. The tumor volume was serially measured by magnetic resonance (MR) imaging with gadolinium as the enhanced area, and the survival periods and histological findings were also examined. Separately, to examine the effects of intracerebral carboplatin infusion on vascular permeability, tumor-bearing rats received intravenous administration of 2% Evans blue at 21 days after infusion. The high dose group showed transient increase of enhanced volume at 21 days associated with mass effect, and significantly decreased tumor volume at 28 and 35 days compared with the control and low dose groups. The high dose group showed significant longer survival time than the control and low dose groups. Histological examination of the high dose group at 21 days showed the central tumor necrotic area around the infusion site and Evans blue leakage into the surrounding enhanced rim and the necrotic core. Therefore, leakage of plasma fluid into the necrotic area was considered to be the cause of apparent transient swelling. The present study demonstrated quantitatively using MR imaging that intracerebral carboplatin microinfusion significantly inhibited the rapid growth of experimental rat glioma but that the high dose required carries the risk of transient swelling of the target tumor.

Key words: intracerebral microinfusion, carboplatin, chemotherapy, brain tumor, rat model

Introduction
Malignant gliomas are invariably fatal, and all efforts to develop new diagnostic and therapeutic modalities have failed to establish a curative regimen. Common chemotherapeutic protocols have failed to extirpate malignant gliomas under clinical conditions, despite significant effects on glioma cells in many laboratory studies.2,5) Malignant gliomas almost always spread locally along the neuronal fibers in the brain, and rarely form metastatic lesions outside the central nervous system, so limited intracerebral infusion would be the most effective approach to delivery of chemotherapeutic agents.

Intracerebral microinfusion is an innovative local drug delivery system which injects therapeutic compounds directly into the brain parenchyma via a subcutaneously implanted osmotic pump.12) This technique provides more homogeneous and greatly extended regional distribution of a chemotherapeutic agent driven by the pressure gradient of the solution, and can achieve drug concentrations in the brain orders of magnitude greater than systemic levels,2,17) in contrast with the distribution by diffusion-driven deliveries such as biodegradable polymers and carmustine wafers,2,11,18) Intracerebral microinfusion also bypasses vulnerable organs outside the central nervous system such as the bone marrow, the mucosa of the digestive tract, and the hair follicle cells, which are all vulnerable to cytotoxic agents because
of the large populations of mitotic cells. In contrast, neural tissue shows no aggressive mitotic activity after birth, so intracerebral microinfusion can be regarded as a less toxic and more efficient method of administration.\(^\text{6,14}\)

The advantages and disadvantages of this technique must be thoroughly investigated before application to clinical practice. The beneficial implications concerning survival elongation have already been reported.\(^\text{6,12}\) However, the solute concentration for adequate antitumor activity, the infusion volume sufficient to prevent tumor spread, and the fluid dynamics in the intracerebral environment affected by the tumor are still unclear.\(^\text{6,14}\)

The present study investigated the drug distribution and efficacy of carboplatin administered by intracerebral microinfusion, and the temporal profile of vascular permeability in malignant glioma-bearing rats treated with two different concentrations of carboplatin using osmotic mini-pumps.

### Materials and Methods

9L/LacZ glioma cells (American Type Culture Collection, Manassas, Va., U.S.A.) were maintained at 37°C in 5% CO\textsubscript{2} in Dulbecco’s modified Eagle medium with 10% fetal bovine serum. On the day of tumor cell inoculation, the cells were trypsinized briefly with 0.25% trypsin with ethylenediaminetetra-acetic acid and suspended in the medium. The suspension was then centrifuged at 1,000 rpm for 3 minutes. The centrifuged cell pellet was resuspended in phosphate-buffered saline (PBS) to a concentration of 20,000 cells/\mu l and kept on ice until inoculation.

The rat glioma model was induced in male F344/N \textit{Slc} Fischer rats weighing 220 to 260 g (Japan SLC, Inc., Hamamatsu, Shizuoka). Animal tests and housing were administered in accordance with the Japanese Association for Laboratory Animal Science Guidelines on the Care and Use of Animals and the animal study protocol was approved by the Juntendo Casualty Center Animal Care and Use Committee. The rats were fixed in a stereotaxic frame (Narishige Scientific Instrument Lab., Tokyo) under light anesthesia (2–3% halothane in 70% N\textsubscript{2}O/30% O\textsubscript{2}). 9L/LacZ glioma cells were inoculated as described previously.\(^\text{15}\) Briefly, after midline scalp incision, a 1 mm diameter hole was made in the left frontal bone with an air drill. Using a 10-\mu l Hamilton syringe with a 25 G needle, 100,000 cells suspended in 5 \mu l PBS were stereotactically injected over 5 minutes through an entry site located 1 mm anterior to the bregma, 2 mm left lateral to the midline, and 5 mm below the surface of the skull. Two weeks after the inoculation, all rats were examined by magnetic resonance (MR) imaging with intravenous administration of gadolinium under anesthesia using intraperitoneal injection of pentobarbiturate (50 mg/kg) to confirm the growth of the inoculated tumor in the brain before pump implantation.

Alzet osmotic mini-pumps (1 \mu l/hr, 7-day infusion capacity) and brain infusion kits consisting of polyvinyl chloride tubing, cut to 3.5 cm, and attached to an infusion cannula (DURECT Corp., Cupertino, Calif., U.S.A.) were assembled according to the manufacturer’s instructions. Carboplatin (cis-diammine(1,1-cyclobutane dicarboxylato)platinum; Sigma-Aldrich Chemical Corp., St. Louis, Mo., U.S.A.) was dissolved in 5% D-glucose on the day of infusion. The agent is stable under the infusion condition for over 4 weeks.\(^\text{19}\) The drug-loaded pump was implanted subcutaneously on the back of the anesthetized rat between the scapulae, and the infusion cannula was inserted into the rat cerebrum through the burr hole previously drilled for tumor cell inoculation using the stereotaxic frame, at the same coordinates as the theoretical center of the tumor mass, and the cannula was secured with glue. The skin incision was closed with surgical sutures.

To evaluate drug distribution in glioma-bearing rat brains, 6 rats were implanted with osmotic mini-pumps loaded with 0.5% Evans blue dissolved in 5% D-glucose 2 weeks after tumor cell inoculation. The animals were examined by MR imaging after 3-day or 7-day infusion (3 rats each), and then euthanized with excess anesthesia. The brains were removed and fixed in 10% formaldehyde. Coronary sections were photographed and stained to compare the extent of the brain tumor and the Evans blue distribution.

To evaluate the effects of intracerebral infusion of carboplatin, 24 glioma-bearing rats were assigned to three groups, 0.1 mg/ml carboplatin (low dose group, 8 rats), 1.0 mg/ml carboplatin (high dose group, 8 rats), and 5% D-glucose (control group, 8 rats). Two weeks after tumor inoculation, the loaded mini-pump was implanted in the rats and the tumor and surrounding regions were infused at 1 \mu l/hr. Upon completion of the 7-day infusion, the empty pumps were removed from the animals under halothane anesthesia. Rats were observed on a daily basis until Day 100, and all surviving rats underwent MR imaging weekly until the 5th week after tumor inoculation. Tumor volume was measured by radiological technicians unaware of the protocol of this study. Surviving animals were euthanized at 100 days (considered to be long-term survival). The brains were harvested from the rats and fixed in 10% formaldehyde after euthanization or at the time of the study.

*Neurol Med Chir (Tokyo) 49, December, 2009*
death. Drug efficacy was analyzed according to survival duration, the findings of serial MR images, and histological findings with hematoxylin and eosin staining.

To evaluate the effect of carboplatin intracerebral microinfusion on cerebrovascular permeability in a tumor-harboring brain, animals were injected with 5 ml/kg Evans blue in normal saline (2%) via the tail vein after removing the empty pumps and MR imaging with gadolinium on completion of 7-day infusion of carboplatin or 5% D-glucose into tumor-bearing brains. One hour after the injection, the animals were perfused with approximately 100 ml of 10% formaldehyde through the left ventricle, and the brains were removed and sectioned. The pattern of Evans blue staining in the brains was compared with the MR images and the histological findings of each rat.

Data are presented as means ± standard errors. Differences in tumor size between groups were determined by Turkey’s pairwise comparisons test. The Kaplan-Meier method was used for plotting animal survival, and differences were analyzed with the log-rank test. P values < 0.05 were considered statistically significant.

Results

Modeling of drug distribution using 0.5% Evans blue infusion in tumor-bearing rat brain showed adequate extension of the dye during both 3-day and 7-day infusion to cover the entire tumor, and into the contralateral hemisphere along the corpus callosum (Fig. 1).

MR images of the representative rats in the low and high dose and control groups are shown in Fig. 2. The rats in the control group showed rapid enlargement of the homogeneously enhanced mass. The rats in the low dose carboplatin group also showed homogeneously enhanced mass but growth was relatively slow compared with the control group. The rats in the high dose carboplatin group showed ring-like enhancement in the topical region after treatment (Days 21 and 28), which had disappeared by the end of the study (Day 100) resulting in long-term survival. The ring-like enhanced mass showed mass effect with shift of the midline structures on Day 21, and some rats suffered obstructive hydrocephalus.

The temporal profiles of enhanced volume are shown in Fig. 3. The high dose carboplatin group had significantly larger enhanced volume on Day 21, which was the last day of infusion, compared with both the low dose group and the control group (p < 0.05). This observation suggested that high dose carboplatin infusion resulted in transient volume expansion during the treatment. The high dose group showed significant smaller volume on Day 28, compared with the control group (p < 0.05), whereas the low dose group showed modest volume reduction. Significantly smaller enhanced volume was observed on Day 35 in both the high dose (p < 0.01) and low dose (p < 0.05) groups compared with the control group.

The median Kaplan-Meier survival time was 30.5 days (range 28–42 days) in the control group, 33 days (range 30–60 days) in the low dose group (p = 0.0864), and 60 days (range 51–100 days) in the high dose group (p < 0.01) as shown in Fig. 4. Three of 8 rats receiving high dose carboplatin infusion survived the entire observation period (100 days), and were considered long-term survivors.

The largest cross-sectional area of tumor occurred in the control group (Fig. 5A). The tumor masses were considered large enough to kill the rats soon after tumor cell inoculation. Some of the rats in the low dose group showed small necrotic areas but the brains were occupied by a large tumor mass like those of the control group (Fig. 5B). Histological examination revealed no evidence of tumor growth in the rat brains that attained long-term survival, but a small cavity surrounded by gliosis was noted (Fig. 5C).

Representative T1-weighted MR images with gadolinium and fluid-attenuated inversion recovery (FLAIR) MR images, and the corresponding coronal
Fig. 2  T₁-weighted magnetic resonance images with gadolinium on Days 14, 21, 28, 35, and 100 (from left to right) after tumor inoculation showing the rapid enlargement of homogeneously enhanced mass in the control group (A), relatively gradual growth of the homogeneously enhanced mass in the low dose group (B), and ring-like enhancement in the topical region after treatment (Days 21 and 28), associated with mass effect with midline shift and obstructive hydrocephalus on Day 21, which disappeared by the end of the study (Day 100) in the high dose group (C).

Fig. 3  Temporal profiles of enhanced tumor volume. Statistically significant differences are indicated as *p < 0.05 or **p < 0.01. The error bars represent the standard error of the mean. Open circles: control group, closed triangles: low dose carboplatin group, closed circles: high dose carboplatin group.

Fig. 4  Kaplan-Meier survival fraction curves for each group in the efficacy study. Open circles: control group, closed triangles: low dose carboplatin group, closed circles: high dose carboplatin group.

Intracerebral Microinfusion of Carboplatin

brain sections after intravenous administration of Evans blue in the low dose and high dose carboplatin groups at completion of infusion are shown in

Neurol Med Chir (Tokyo) 49, December, 2009
Fig. 5 Photographs of representative coronal brain sections from each group (left column) and photomicrographs (right column; hematoxylin and eosin stain, original magnification ×50) showing a large tumor mass in the cerebral hemisphere with uniformly compacted tumor cells in the control group (A), a similar large tumor mass with necrotic islands in the low dose group (B), and a small cavity surrounded by gliotic tissue with no evidence of residual tumor cells in the high dose group with long-term survival (C).

The present study demonstrated adequate distribution of 0.5% Evans blue to cover the entire tumor in tumor-harboring rat brains, which indicates that continuous intracerebral microinfusion using the osmotic mini-pump can provide broader distribution of agents than growth of the brain tumor. Moreover, the solution also reached the contralateral hemisphere via the corpus callosum and tracked along neuronal fibers mimicking tumor spread, which is definitely an important advantage of this modality for preventing dissemination of tumor cells. The present study also showed that intracerebral microinfusion can attain clinically favorable drug distribution from a single infusion point.

The present study of drug efficacy used 5% D-glucose as control and two different concentrations of carboplatin were employed to evaluate the therapeutic impact on brain tumor-bearing animals. A previous toxicity study of carboplatin using short-time intracerebral infusion tested 0.1, 1.0, and 10 mg/ml doses. None of the animals infused with 0.1 or 1.0 mg/ml of carboplatin demonstrated any clinical signs of toxicity or had histological evidence of toxicity, whereas all four rats infused with 10 mg/ml of carboplatin died within 3 days. Therefore, the doses of carboplatin in this study, 1.0 mg/ml as a high dose and 0.1 mg/ml as a low dose, were chosen to avoid animal death caused by drug toxicity.

The control group showed rapid enlargement of homogeneously enhanced volume on MR imaging, indicating rapid tumor growth, and all rats died within 42 days. In contrast, both low and high dose groups showed a trend of longer survival period and significantly smaller enhanced volume on MR imaging after 21 days. Three of 8 rats in the high dose carboplatin group survived for more than 100 days, and histological examination of their brains disclosed complete disappearance of the tumor, resulting in a small cavity surrounded by gliosis. This finding illustrates the striking impact of carboplatin microinfusion on the treatment of intracerebral malignant tumors. The low dose carboplatin group showed longer median survival, but did not reach statistically significant advantage compared with the control group.

Histological examination also demonstrated that all rats in the low dose and control groups harbored tumor masses in the cerebral hemisphere at the time.
Fig. 6  $T_1$-weighted magnetic resonance (MR) images with gadolinium (left column), fluid-attenuated inversion recovery (FLAIR) MR images (center column), and photographs of the corresponding coronal brain sections after intravenous administration of 2% Evans blue (right column) showing the homogeneously enhanced area that completely correlated with the area of Evans blue staining in the low dose group (A), ring-like enhancement with the core appearing hyperintense on FLAIR images in the high dose group (B), and leakage of Evans blue in both the peripheral enhanced area and the central core (A, B).

Fig. 7  Photomicrographs of the same coronal sections A and B in Fig. 6 showing relatively sparse tumor cell density around the infusion cannula (A1) and gradually higher tumor cell density in the peripheral part (A2) in the low dose group, and necrosis in both the central core (B1) and peripheral area (B2) in the high dose group, with dilated capillaries and reactive gliosis in the boundary zone (B2), but no tumor cells in the interface between the necrotic area and the surrounding brain tissue except for the small tumor cell island apart from the carboplatin infusion site (B3, arrow). Hematoxylin and eosin stain, original magnification A1 and A2: $\times 100$ and B1–B3: $\times 50$. 

*Neurol Med Chir (Tokyo) 49, December, 2009*
of death. However, rats in the low dose group had prominent small necrotic areas in the center of the tumor mass. Furthermore, histological examination of the rat brains in the low dose group at 21 days in the vascular permeability study showed relatively sparse tumor cell density around the infusion cannula, and increasingly dense tumor cells in the peripheral part of the tumor mass. This observation indicates the limited therapeutic effect in the peri-infusion areas of the lower concentration of carboplatin.

Carboplatin is an alkylator, which exerts its anti-tumor effects by binding to the deoxyribonucleic acid and producing intra- and interstrand crosslinks during replication. Carboplatin shows potential for treating central nervous system tumors including glioma, and has lower nephrotoxicity and neurotoxicity compared to cisplatin, which is a first generation platinum compound.¹⁻¹⁰,¹⁶ Unlike some chemotherapeutic drugs, carboplatin possesses cytoxic activity without previous metabolization. Moreover, carboplatin is hydrophilic and so does not easily cross the blood-brain-barrier.⁸ Therefore, carboplatin is a good candidate for intracerebral topical administration such as our method. The tumoricidal effect of platinum compounds has been tested in both human and rodent glioma cell lines using various methods. In particular, the in vitro stability and cytotoxicity of cisplatin and carboplatin were evaluated using rat 9L glioma cells, and found that only 2-hour incubation of 90 μg/ml carboplatin with 9L glioma cells resulted in approximately 75% decreased viable cell fraction using 3-(4,5 dimethylthiazol–2-yl)–2,5 diphenyltetrazolium bromide assay compared to no treatment control.⁹ In addition, the transformed median lethal concentration values of chemotherapeutic agents were calculated from accumulated data, and carboplatin levels of 55–75 μg/ml were estimated to inhibit 70% of cell growth in several glioma cell lines in vitro.²⁰ However, none of the rats in our low dose group survived the entire observation period, despite the use of a higher concentration of carboplatin. Therefore, a certain concentration of carboplatin is clearly required to treat intracerebral brain tumors in vivo using intracerebral microinfusion.

The enhanced volume on MR imaging was significantly larger in the high dose group than in the other two groups on Day 21. This temporary increase in mass caused a mass effect on the surrounding brain tissue. The less enhanced core was surrounded by a ring-like enhanced rim. Histological examination showed that the less enhanced core corresponded to amorphous necrotic tissue. The boundary zone between the necrotic core and the surrounding brain tissue contained dilated capillaries with reactive gliosis. Intravenous administration of Evans blue penetrated both the enhanced rim and the necrotic core. These findings suggest that the gadolinium agent and the Evans blue leaked from the blood vessels in the boundary zone.⁷ Leakage of plasma components (edema fluid) from these permeable blood vessels probably flowed into the necrotic core, and so caused the swelling of the tumor. Therefore, the curative dose of carboplatin might carry the risk of temporary worsening of the mass effect in the course of intracerebral infusion therapy.

The present study showed the possibility of complete disappearance of malignant glioma by intracerebral infusion of carboplatin. However, temporary worsening due to mass effect is a risk. Steroid administration may be helpful to suppress the transient swelling caused by the increased vascular permeability. Preclinical data such as optimal concentration, infusion period, total dosage, and neurotoxicity of carboplatin are still needed. We have to be aware of the both beneficial and adverse effects of the intracerebral infusion therapy before its clinical application.

References

Intracerebral Microinfusion of Carboplatin


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Commentary

Carboplatin, the second generation platinum compound, is a classic alkylating agent among antitumor drugs. In the present article, Yuichi Tange et al. performed an investigation for the novel antitumor effect of carboplatin delivered by intracerebral microinfusion in a rat malignant glioma model. Intracerebral microinfusion, an innovative technique of regional delivery of therapeutic agents directly into brain tumor, has the potential to bypass the BBB and vulnerable organs leading to high local concentration, less toxicity, and more effectiveness. Compared with other methods of delivering therapeutic agents directly into an intracerebral tumor such as drug-impregnated polymers, direct bolus infusions, and intrathecal or intraventricular infusion which highly dependent on the molecular weight of the agent, intracerebral microinfusion uses a pressure gradient to produce a bulk flow infusion in a more homogeneous fashion over a greater area. Such enhancement of drug distribution has been demonstrated in experimental animal models and preliminary human trials. This is a valuable study with potential clinical implications. The authors concluded that intracerebral carboplatin microinfusion significantly inhibited the rapid growth of experimental rat glioma but that the high dose required the risk of transient swelling of the target tumor. In my opinion, the point of the future study is to optimize drug concentration, to define the pharmacokinetics and distribution, and to attenuate the temporary brain swelling and other side effects.

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