Combination Therapy of Surgical Tumor Resection with Implantation of a Hydrogel Containing Camptothecin-Loaded Poly(lactic-co-glycolic acid) Microspheres in a C6 Rat Glioma Model

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We have developed a drug-loaded poly(lactic-co-glycolic acid) (PLGA) microsphere-containing thermoresversible gelation polymer (TGP) (drug/PLGA/TGP) formulation as a novel device for implantation after surgical glioma resection. TGP is a thermosensitive polymer that is a gel at body temperature and a sol at room temperature. When a drug/PLGA/TGP formulation is injected into a target site, PLGA microspheres in TGP gel localize at the injection site and do not diffuse across the entire brain tissue, and thus, sustained drug release from the PLGA microspheres at the target site is expected. Using in vivo imaging, we confirmed that the implantation of indocyanine green (ICG)/PLGA/TGP formulation exhibited a stronger localization of ICG at the injection site 28 d after injection compared with that of ICG/PLGA formulation. The therapeutic effect (mean survival) was evaluated in a C6 rat glioma model. Surgical tumor resection alone showed almost no effect on survival (controls, 18 d; surgical resection; 18.5 d). Survival was prolonged after the treatment with a camptothecin (CPT; 10 µg)/PLGA/TGP formulation (24 d). The combination treatment of surgical tumor resection and CPT/PLGA/TGP showed almost the same therapeutic effect (24 d) compared with CPT/PLGA/TGP alone, while the combination treatment produced long term survivors (>60 d). Therefore, the CPT/PLGA/TGP formulation can be an effective candidate for localized and sustained long-term glioma therapy.

Key words glioma therapy; surgical tumor resection; sustained release; poly(lactic-co-glycolic acid) microsphere; thermoresversible gelation polymer

Glioblastoma multiforme (GBM) is a lethal brain tumor and the most common and malignant of the glial tumors. Despite aggressive surgical resection, radiation therapy, and chemotherapy, the prognosis of GBM remains poor: the survival rate 2 years after surgery is only 5–10%.1–4 Therefore, new therapeutic strategies to inhibit recurrent GBM after surgical resection are needed. Local delivery of chemotherapeutic drugs is a promising approach for the treatment of brain tumors.5–8 Direct delivery of chemotherapeutic drugs to brain tumors bypasses the blood–brain barrier (BBB) and can achieve high concentrations of the drug at target site with minimized risk of systemic toxicity.

As a potential strategy for glioma therapy based on local delivery, we previously developed a novel device for the treatment of brain tumors using the implantation of a formulation consisting of a unique thermoresversible hydrogel containing drug/poly(lactic-co-glycolic acid) (PLGA) microspheres.9 Thermoresversible gelation polymer (TGP) hydrogel (MebiolTM Gel) is a gel at body temperature and a sol at room temperature.10–13 Therefore, when the formulation (drug/PLGA microspheres dispersed in TGP sol) is injected into the target site, TGP is thought to form a gel around the injection site, retain the drug/PLGA microspheres, and not diffuse across the entire brain tissue. PLGA is a biodegradable polymer that has been used as a base for the delivery of long-term sustained-release microspheres.14–17 Therefore, our formulation allows the drug to be released at the target site continually and efficiently.

In this study, we evaluated the combination therapy of surgical resection with a camptothecin (CPT)/PLGA microsphere-containing TGP (CPT/PLGA/TGP) formulation. CPT, which inhibits DNA topoisomerases, has a potent antitumor effect on glioma cell lines and the CPT derivatives have been utilized for patient with glioma.18–20 Previously, we evaluated the CPT/PLGA/TGP formulation in a C6 rat glioma model.9 In the present study, we developed a surgically removed tumor model that is expected to be closer to clinical use and the therapeutic efficacy of the CPT/PLGA/TGP formulation was investigated.

MATERIALS AND METHODS

Materials CPT and PLGA (75:25 lactic acid/glycolic acid, molecular weight (MW)=4400) were purchased from Wako (Osaka, Japan). Indocyanine green (ICG) was purchased from Kanto Chemical (Tokyo, Japan). TGP (MebiolTM Gel) was supplied by Mebiol Inc. (Kanazawa, Japan). All other reagents were of analytical grade.

Preparation of Drug/PLGA Microspheres ICG-loaded and CPT-loaded PLGA microspheres (ICG/PLGA and CPT/PLGA) were prepared by the water-in-oil-in-water emulsion technique as previously described.9 In brief, 200 µL of 0.4% aqueous polyvinyl alcohol containing 10 mg of the drug (ICG or CPT) was added to 3 mL dichloromethane containing 500 mg PLGA (the ratio of drug to PLGA was 1:50 w/w). The mixture was homogenized with a Polytron PT 3100 (Kinematica AG, Switzerland) at 10000 rpm for 3 min, and the resulting drug/PLGA emulsion was added to 300 mL of 0.25% aqueous polyvinyl alcohol. The final mixture was homogenized with a TK Homo Mixer Mark II (Tokushu Kika,
Hyogo, Japan) at 3000rpm for 3 min, and the resulting drug/PLGA/polyvinyl alcohol emulsion was stirred gently for 3 h to evaporate the dichloromethane. The drug/PLGA microspheres were centrifuged at 880×g for 15 min, rinsed three times with distilled water, lyophilized, and stored at −80°C until use. The suspension of drug/PLGA microspheres was prepared by adding an appropriate amount of water before use.

Characterization of Drug/PLGA Microspheres Using Scanning Electron Microscopy (SEM) The structures of ICG/PLGA and CPT/PLGA microspheres were observed using an S-2250N scanning electron microscope (Hitachi, Tokyo, Japan) as previously described. In brief, the microparticles were coated with 25-nm-thick gold using a quick carbon coater (SC-701; Sanyu Electronics, Tokyo, Japan). The diameter (horizontal Feret’s diameter) of the microspheres was measured using WinROOF image analysis software (Mitani, Fukui, Japan). The mean particle diameter was defined as the median diameter of the cumulative curve of the number-based particle size distribution.

High Performance Liquid Chromatography (HPLC) The concentration of CPT was determined by HPLC (Shimadzu Class LC-10 series) as previously described. In brief, the system consisted of a pump (Shimadzu LC-10AT), detector (Shimadzu SPD-10A), control module (Shimadzu CBM-10A), an auto-injector (Shimadzu SIL-10XL), column oven (Shimadzu DGU-12A), and column, (Mightysil RP-18, 4.6 mm×150 mm; Kanto Chemical, Tokyo, Japan). The flow rate was 1.0 mL/min and the column temperature was maintained at 40°C. A 3:6:1 5 mM KH2PO4–5 mM K2HPO4–acetonitrile–methanol solution (v/v/v) was used as the mobile phase. The analysis was performed with UV detection at 254 nm.

TGP TGP comprises conjugates of polyethylene glycol and poly(N-isopropylacrylamide) which is a thermoreversible polymer consisting of N-isopropylacrylamide and n-butyl methacrylate. The unique temperature kinetics of TGP have been previously reported. At low temperatures (<32°C), amide groups are hydrated, which converts the polymer into the sol state. At high temperatures (around body temperature), the hydrogen bonds between the amide groups and water molecules dissociate and the polymer chains aggregate because of the hydrophobic interactions mediated by the isopropyl groups, causing the polymer to transition to the gel state. To prepare 10% w/v aqueous solution, 1 g TGP powder was dissolved in water, and then adjusted to 10 mL. The TGP solution was stored at 4°C until use. The TGP solution was directly added the prepared drug/PLGA microspheres (lyophilized powder as described in 'Preparation of Drug/PLGA Microspheres'), and then, the drug/PLGA/TGP solution was prepared.

Animals and Tumor Cell Lines C6 rat glioma cells were purchased the European Collection of Cell Cultures (Salisbury, U.K.). The cells were maintained in Ham’s F-12 medium (Invitrogen, CA, U.S.A.), supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 1 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen). The cells were incubated at 37°C in a humidified atmosphere of 5% CO2 and 95% air.

Male Sprague-Dawley rats (6 weeks old, 180–220 g) were purchased from Japan SLC (Shizuoka, Japan). The animals had free access to food and water. The experiments were performed according to the Guidelines of Experimental Animal Care issued by the Prime Minister’s Office of Japan. The experimental protocol was approved by the Committee of Animal Care and Use of the Tokyo University of Pharmacy and Life Sciences.

Rat Glioma Models The rat glioma models were prepared as previously described. In brief, the rats were anesthetized and their heads were fixed in position with a brain stereotaxic instrument (SR-6R; Narishige, Tokyo, Japan). A part of the hair scalp was removed with an electric hair clipper. The skin of the scalp was then cut out along the midline of the head with surgical scissors, and the cut area was expanded with cotton swabs. A point 3 mm to the left and 3 mm forward from lambda of the bone of the skull was marked. A hole was created in the skull with a drill (Proxxon mini router and drill bit, external diameter (ϕ)=0.5 mm; Kiso Power Tool, Osaka, Japan) at the marked point. A 30-gauge needle with microsyringe was inserted at a depth of 7 mm from the skull surface, and 10 µL of a C6 cell suspension (1×10^6 cells) was injected at 3.33 µL/min using a microsyringe pump (CFV-2100; Nihon Koden, Tokyo, Japan). The needle was kept in position for 5 min and then withdrawn over a period of 3 min to prevent suspension from flowing backwards. After the injection, the hole in the skull was covered with bone wax (Luxens; Surgical Specialties, PA, U.S.A.). The skin on the scalp was closed using a suture needle (Natsume Seisakusho, Tokyo, Japan) and sterilized with isodine (Meiji Seika, Tokyo, Japan). Seven days after the tumor inoculation, treatment was initiated.

In Vivo Imaging of ICG/PLGA/TGP Gel in the Brain Tissue of a in Tumor-Free Rat The ICG/PLGA or ICG/PLGA/TGP formulation was locally administered to the target site in a tumor-free rat as described in ‘Rat Glioma Models.’ The formulation (30 µL) was injected into the site at 10 µL/min. After the injection, the hole in the skull was sealed with bone wax (Luxens; Surgical Specialties). The scalp skin was sutured and sterilized with isodine (Meiji Seika, Japan). Twenty-eight days after implantation of the ICG/PLGA/TGP formulation, the rat was sacrificed, and brain tissue was collected. The brain tissue was monitored with a Maestro in vivo imaging system (excitation: 470–565 nm; emission: 700 nm long-pass; CRI Inc., MA, U.S.A.), and images were obtained.

Surgical Tumor Resection in the C6 Rat Glioma Model Seven days after C6 cells were inoculated (‘Rat Glioma Models’), a hole was again created with a wider drill (Proxxon mini router and drill bit, ϕ=1.2 mm; Kiso Power Tool) at the same point. A needle for the isolation of the hypophysis (ϕ=1.5 mm; Natsume Seisakusho, Japan) with an aspirator was inserted at a depth of 6 mm from the skull surface. The needle was kept in position for 5 s and the tumor was isolated. After isolation, the hole in the skull was covered with bone wax (Luxens; Surgical Specialties). The skin on the scalp was closed using a suture needle (Natsume Seisakusho) and sterilized with isodine (Meiji Seika).

Therapeutic Effect of Surgical Resection and/or CPT/PLGA/TGP in C6 Rat Glioma Model After establishing the rat glioma model and the surgically removed tumor model (‘Rat Glioma Models’ and ‘Surgical Tumor Resection in the C6 Rat Glioma Model’), different formulations were locally administered to the target sites in the models. The formulations (30 µL) were injected into the brain at 10 µL/min. The rats were separated in six groups: Group 1, controls (untreated rats); groups 2 and 3, treatment with CPT (10 or 30 µg)/PLGA/TGP.
The therapeutic effects were evaluated by determining the survival rates of the groups. The Kaplan–Meier method was used to estimate survival, and survival differences were analyzed by the log-rank test. A value of $p < 0.05$ was considered statistically significant. Whole body weights were simultaneously monitored as a measure of systemic toxicity.

Hematoxylin and Eosin (H&E) Staining and the Determination of Tumor Size

Rat brain tissues were collected, and then, the tissues were soaked in 70% ethanol. Tissue blocks were then processed in an automatic tissue processor (Tissue-Tek VIP; Miles Scientific, IN, U.S.A.) and embedded in paraffin. Tissue blocks were sectioned at 20 µm and the sections were deparaffinized by completely submerging the slide in xylene three times for 10 min and were further stained with H&E. The H&E protocol was following the treatment of tissue sections sequentially in 100, 95, 80, 70% ethanol, water and hematoxylin (Wako) and eosin (Wako). Stained sections were observed by light microscopy. The size of the tumor was represented the formula (1) as described below and was the tumor region (deeper colored part) was measured on the images of the H&E section.

$$\text{tumor volume (mm}^3\text{)} = \frac{1}{2} \ \text{length} \times \text{width}^2 \quad (1)$$

RESULTS

Physicochemical Characteristics of Drug-Loaded PLGA Microspheres

SEM images of the drug/PLGA microspheres are shown in Fig. 1. All particles were nearly spherical, and the mean diameters of the ICG/PLGA and CPT/PLGA microspheres were 28.0±13.7 µm and 39.3±20.0 µm, respectively (Table 1). The encapsulation efficiency of the CPT/PLGA microspheres was 30.1±9.1%.

Effects of TGP on Retention of the Drug/PLGA Microspheres at the Targeted Site

After injection of the drug/PLGA/TGP sol into the target site, we expected the PLGA microspheres in the TGP gel to localize at the injection site with minimum diffusion across the entire brain tissue. Using in vivo imaging analysis, we confirmed whether the drug/PLGA microspheres localized at the target site (Fig. 2). When the ICG/PLGA formulation without TGP was injected into the brain of the tumor-free rat, ICG dye was faintly observed at the injection site in the bright-field images (Fig. 2A upper panel): however, the fluorescence was clearly observed at the injection site and was seen to be diffused throughout the upper site (Fig. 2A lower panel). This meant that the PLGA formulation flowed back away from the injection site. In comparison, when the ICG/PLGA/TGP formulation was injected into the rat brain tissue, ICG was strongly localized at the injection site (Fig. 2B upper panel), and an even stronger fluorescence was localized at the injection site 28 d after the injection of formulations (Fig. 2B lower panel). Flow back of the injected formulation was minimal.

Tumor Growth after Surgical Removal of the Glioma

A strongly stained tumor was observed in the hematoxylin-eosin stained slices of the glioma from the rat brain 7 d after stereotactic implantation of the C6 glioma cells (Figs. 3A,D). After the surgical tumor resection, the tumor apparently disappeared (Fig. 3B), but some remaining tumor was observed at high magnification (Fig. 3E), which meant that complete removal of the tumor was difficult to achieve. Seven days after removal of the tumor, it had reoccupied the brain tissue (Figs. 3C,F). The tumor volume was simultaneously measured (Fig. 4). Five days after the tumor inoculation, no tumor was observed. Tumor growth was observed to begin at 7 d, and a large amount of brain tissue was occupied with tumor by 14 d after the tumor inoculation. When the tumor was surgically removed 7 d after tumor inoculation, tumor regrowth was observed 7 d after the surgical removal (14d after tumor inoculation).

Evaluation of Therapeutic Effects by Injection of the Drug/PLGA/TGP Microspheres in the Rat Brain Tumor
The therapeutic effects were evaluated in terms of survival (Fig. 5, Table 2). The treatment with surgical tumor resection alone had almost no effect on survival (untreated control rats; 18 d: surgical resection treatment; 18.5 d). In contrast, the treatment with CPT/PLGA/TGP formulation exhibited significant survival (10 µg/rat CPT; 24 d: 30 µg/rat CPT; 26 d) compared with the untreated rats. However, the combination of surgical resection plus treatment with the CPT/PLGA/TGP formulation and treatment with the CPT/PLGA/TGP alone had similar therapeutic effects on survival (10 µg/rat CPT; 24 d: 30 µg/rat CPT; 26 d). Remarkably, two of seven rats that underwent combination therapy were long-term survivors (>60 d). No remarkable body weight changes were observed in any of the rats after the treatment with the formulations (data not shown).

**DISCUSSION**

Radical surgery is the primary therapeutic strategy for glioma; however, the therapeutic benefit are low because of recurrence of malignancy. In GBM, it is difficult to remove the glioma completely and the tumor has poorly defined borders due to the invasion of normal brain tissue. New therapeutic strategies are required for GBM. We developed a novel local drug delivery system that involves implantation of a drug/PLGA/TGP formulation after surgical tumor resection. The drug/PLGA/TGP formulation can be easily implanted into the target site at the time of surgery and the resection cavity after removal of the tumor can be used for the implantation. In addition, the strategy allows delivery of high drug concentrations and bypass of BBB and minimizes the risk of systemic toxicity. Therefore, the combination of surgical treatment and
the local drug delivery system of drug/PLGA/TGP formulation can be an effective and reasonable strategy for glioma therapy.

In this study, we used the combination of TGP and PLGA to develop an efficient localized and sustained drug delivery system. TGP is a sol at room temperature and a gel at body temperature. This property allows TGP to be injected into the target site and form a gel around the target site. In this study, drug-loaded PLGA microspheres were dispersed in TGP sol. PLGA is a biodegradable and controlled-release polymer and has been used for long-term delivery of several therapeutic peptides and also for low-molecular weight drugs.\textsuperscript{14–17} With the system, the PLGA microspheres release drugs at the target site and do not diffuse drugs across the brain tissue because they are retained in the localized TGP gel. The fluorescence from ICG in the ICG/PLGA/TGP formulation was observed to remain localized for 28d after the injection. The fluorescence from the ICG/PLGA/TGP formulation was stronger compared with that from the ICG/PLGA solution (Fig. 2). These results suggested that the drug/PLGA/TGP formulation was effective as a local drug delivery system. In our previous study, fluorescence-labeled PLGA microspheres also showed localization at the target site after 1 month,\textsuperscript{9} which is consistent with our present results. Our new finding from this study is that the ICG/PLGA formulation without TGP was observed to flow back from the injection site; however, this was not observed with the ICG/PLGA/TGP formulation. These results suggested that our formulation may be useful in preventing an injection side effect. The drug/PLGA/TGP formulation appeared to be a suitable device for localized and sustained drug delivery in GBM therapy.

Complete tumor removal by surgical treatment alone is very difficult because the glioma can invade the surrounding normal tissue. Using microscopy under high magnification, we found that some tumor was left after the surgical resection (Fig. 3B). The growth of tumor after the surgical resection is similar or sometimes even faster compared with that of primary tumors.\textsuperscript{23} Survival time is an indicator of brain tumor growth rate. In the present study, there was little difference in survival between the CPT/PLGA/TGP formulation-treated groups with and without surgical resection (Fig. 5, Table 2). One explanation for the faster tumor growth after surgical resection could be related to the resection cavity. Recurrent tumors can grow more easily because the tumor mass can occupy the resection cavity again without pressure from the surrounding brain tissue. Therefore, incomplete and transient removal by surgery may not result in therapeutic efficacy. In addition, the tumor growth rate in experimental rodent models is generally faster compared with practical cases: however, surgical resection is necessary for glioma therapy against patient and the surgery clearly can improve the survival rate significantly. Furthermore it is hard to kill tumor masses completely by using CPT/PLGA/TGP formulation only (there was no long-term survivor, Table 2). Thus, the combination of surgical removal is important to treat brain tumor.

On the contrary, we found that the implantation of our drug/PLGA/TGP formulation after surgical resection produced some long-term survivors (Fig. 5, Table 2) and the treatment with the surgery or CPT/PLGA/TGP formulation alone did not. These results indicated that the combination of surgical tumor resection with the CPT/PLGA/TGP formulation may eliminate tumor cells. The sustained drug release may be effective for complete elimination of tumor cells that remain after resection. Our prepared drug was released gradually over a long-term in vitro; approximately 70% of the drug was

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**Fig. 4.** Effects of Surgical Tumor Resection on Changes in Tumor Size in the C6 Rat Glioma Model

At each time point, the rats were sacrificed and the brain tissues were collected. Tumor size was measured as described in Materials and Methods. Tumor resection was carried out at day 0. Tumor size was measured at day −7, −4, −2, 0 and 7. Each point represents mean±S.D. (n=5).

**Fig. 5.** Kaplan–Meier Survival Curves of the C6 Glioma-Inoculated Rats after Different Treatments

The treatment protocol was described in Materials and Methods.
Table 2. Median Survival of the Rats after Different Treatments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Median survival (days)</th>
<th>Number of long-term survivors (&gt;60d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>18</td>
<td>0/7</td>
</tr>
<tr>
<td>Surgically removed</td>
<td>18.5</td>
<td>0/10</td>
</tr>
<tr>
<td>Surgically removed+ CPT (10 µg)/PLGA/TGP</td>
<td>24</td>
<td>2/7</td>
</tr>
<tr>
<td>Surgically removed+ CPT (30 µg)/PLGA/TGP</td>
<td>26**</td>
<td>2/7</td>
</tr>
<tr>
<td>CPT (10 µg)/PLGA/TGP</td>
<td>24*</td>
<td>0/5</td>
</tr>
<tr>
<td>CPT (30 µg)/PLGA/TGP</td>
<td>26**</td>
<td>0/5</td>
</tr>
</tbody>
</table>

The survival period of long-term survivors was regarded as 60 d. The survival differences from Kaplan-Meier methods were analyzed by the log-rank test. Asterisk represents the statistically significance (*p<0.05, **p<0.01) compared with control group.

released within 1 month. These results support that the CPT/PLGA/TGP formulation has sustained- and controlled-release properties. In addition, in our previous study, we found that the CPT/PLGA formulation without TGP reduced therapeutic efficacy. This may have been because the CPT/PLGA microspheres diffused across the brain tissue in contrast to the CPT/PLGA/TGP formulation, suggesting that targeted drug release over an appropriate period is essential for maintaining the drug concentration at the target site sufficient for achieving therapeutic effects.

CONCLUSION

Our developed CPT/PLGA/TGP formulation showed the significant therapeutic effect in the experimental glioma model and in the surgical tumor resection model. This drug/PLGA/TGP formulation localized at the injection site for at least 28 d. Since there was no significance between the CPT/PLGA/TGP formulation alone and the combination treatment with CPT/PLGA/TGP formulation and surgical tumor resection, further study is necessary to improve therapeutic efficacy. However, the CPT/PLGA/TGP formulation can be a promising localized and sustained drug delivery system for glioma therapy.

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