Treatment of Rat Brain Tumors Using Sustained-Release of Camptothecin from Poly(lactic-co-glycolic acid) Microspheres in a Thermoreversible Hydrogel

Tetsuya OZEKI,*a Kosuke HASHIZAWA,b,† Daiki KANEKO,b Yoshihiro IMAI,b and Hiroaki OKADA,b

Graduate School of Pharmaceutical Sciences, Nagoya City University; 3–1 Tanabe-dori, Mizuho-ku, Nagoya, Aichi 467–8603, Japan; and b School of Pharmacy, Tokyo University of Pharmacy and Life Sciences; 1432–1 Horinouchi, Hachioji, Tokyo 192–0392, Japan. Received March 16, 2010; accepted June 8, 2010; published online June 17, 2010

A thermoreversible gelation polymer consisting of an aqueous solution in the sol state at room temperature and in the gel state near body temperature was examined for its use in the retention of microspheres and sustained, long-term delivery of anti-cancer drugs using a rat model of malignant glioma. The poly(lactic-co-glycolic acid) (PLGA) microspheres containing camptothecin at ratios of 1 : 33 or 1 : 50 mediated sustained release, with approximate 80% of camptothecin released after 28 d. Rats were inoculated in the brain with C6 glioma cells, followed 7 d later by injection in the tumor site with 1 : 33 and 1 : 50 PLGA microspheres dispersed in a thermoreversible gelation polymer (TGP) solution. Kaplan–Meier analysis showed that the mean survival period of the untreated group was 16 d, with a slight increase in rats treated with TGP-only solution, empty or 1 : 50 microspheres in phosphate-buffered saline. The mean survival period of rats treated with the camptothecin powder in TGP was 21 d, while that of rats treated with 1 : 33 and 1 : 50 microspheres in TGP was significantly longer than the untreated group; long-term survival rates were observed. These results suggest that the anti-tumor effect of camptothecin can be enhanced by long-term sustained release from microspheres retained in the rat brain by TGP gel.

Key words brain tumor; thermoreversible gelation polymer; sustained release; poly(lactic-co-glycolic acid) microsphere; camptothecin

Brain tumors are intracranial masses produced by abnormal and uncontrolled cell division. They develop in the brain itself as well as in the cranial nerves, brain envelopes, skull, pituitary, and pineal glands or arise from metastases of tumors in other organs. Glioma is the most common primary brain tumor and is particularly malignant due to sprouting and spreading within the brain that results in a decline in brain functions, such as language disorders, paralysis on one side of the body, and even death. The most effective treatment for gliomas is thought to be surgical removal, but it is difficult to remove gliomas completely because they have poorly defined borders due to the invasion of normal brain tissue. Chemotherapy is therefore widely used as adjuvant treatment following surgery, but its effectiveness is limited, resulting in its usually poor outcome. In fact, the survival rate two years after surgery is only 5—10% for stage IV glioma.1—8

Direct and continuous long-term injection via a catheter implanted in the skull has been attempted as a way to improve the delivery of anti-cancer drugs to brain tumors, but this is a complicated and risky method.9,10 A system for long-term delivery following a single drug administration is more desirable.

Camptothecin is an alkaloid isolated from Camptotheca acuminata DECNE, a tree species native to China.11 Camptothecin is a time-dependent and potent anti-cancer drug that acts by inhibiting topoisomerase I and thus progression of the S-phase of the cell cycle.12 It was initially studied as a chemotherapeutic agent by the National Institutes of Health in the United States during the 1970s, but it was abandoned due to serious side-effects including suppression of bone marrow and induction of hemorrhagic cystitis.13—16 Since then, various derivatives of camptothecin have been synthesized and assessed in the treatment of cancer.17—21 At the same time, many new drug delivery systems were developed, such as liposomes, polymer micelles, and polymer hybrids.22—30 Here, we examined the use of poly(lactic-co-glycolic acid) (PLGA) microspheres embedded in a thermoreversible gelation polymer (TGP) as a system for the sustained delivery of camptothecin to brain tumors. PLGA is a biodegradable polymer that is metabolized in the body into H2O and CO2. This polymer has been used as a base for the delivery of long-term sustained-release microspheres.31—33

We studied the use of PLGA microspheres dispersed in TGP to achieve the continuous, long-term release of camptothecin in vivo. In this study, PLGA microspheres containing camptothecin were dispersed in TGP in the sol state. Their ability to mediate long-term delivery of camptothecin was examined in a rat model of glioma.

Experimental

Materials Camptothecin, PLGA (75:25 lactic acid/glycolic acid; molecular weight, 14400) and polyvinyl alcohol were purchased from Wako Pure Chemical Industries (Osaka, Japan). Coumarin 6 (excitation and emission wavelengths=505 and 540 nm, respectively) was purchased from Poly-sciences (Warrington, PA, U.S.A.). Rat glioma C6 cells were from the European Collection of Cell Cultures (Salisbury, U.K.). TGP (Mebiol19Ge) was kindly supplied by Mebiol, Inc. (Kanagawa, Japan). Fluorescein isothiocyanate (FITC, excitation and emission wavelengths=493 and 515 nm, respectively) labelled TGP (F-TGP) was also supplied by Mebiol, Inc. All solvents were reagent grade.

TGP TGP consists of conjugates of polyethylene glycol and poly-N-isopropylacrylamide, which is a thermoresponsive polymer consisting of N-isopropylacrylamide and N-butylmethacrylate. N-Isopropylacrylamide includes both hydrophobic isopropyl and hydrogen-bonding amide groups (Fig. 1). The composition ratio of N-isopropylacrylamide (X), N-butylmethacrylate (Y), and polyethylene glycol dimethacrylate (Z) is 97, 2, and 1. The unique temperature kinetics of TGP has been reported in detail by Yoshioka et al.34—37 At low temperatures, the amide groups are hydrated, making the polymer convert to the sol state. At high temperatures, the hydrogen bonds between amide groups and water molecules break, and the polymer chains aggregate due to hydrophobic interactions mediated by the isopropyl groups,
causing the polymer to transition to the gel state (Fig. 2). The temperature of the sol-gel transition, called the lower critical solution temperature (LCST), can be changed by altering the amount of n-butylmethacrylate. In the current study, we used a 10 w/% aqueous solution of TGP with a LCST of 32 °C. Although this solution is in the sol state at room temperature, it changes to the gel state around body temperature, thus keeping the PLGA microspheres in the rat brain.

Preparation of Camptothecin/PLGA Microspheres and Coumarin 6/PLGA Microspheres  The mean diameter of camptothecin was measured to be 2.6 μm with a particle sizer (NICOMP 380 ZLS; Particle Sizing Systems, Santa Barbara, CA, U.S.A.). Camptothecin/PLGA microspheres were prepared by an in-water drying method through a water-in-oil-in-water emulsion.31—33) First, 200 μl of 0.4% aqueous polyvinyl alcohol containing 10 or 15 mg camptothecin was added to 3 ml dichloromethane containing 500 mg PLGA. The mixture was homogenized with a Polytron PT 3100 (Kinematica AG, Switzerland) at 10000 rpm for 3 min and the resulting camptothecin/PLGA emulsion was again added to 300 ml of 0.25% aqueous polyvinyl alcohol. The final mixture was homogenized with a T. K. Homomixer Mark II (Toskush Kika, Hyogo, Japan) at 3000 rpm for 3 min and the resulting camptothecin/PLGA/polyvinyl alcohol emulsion was stirred gently for 3 h to evaporate the dichloromethane. The camptothecin/PLGA microspheres were sedimented by centrifugation at 880×g for 15 min, rinsed three times with distilled water, and lyophilized. The composition ratios of camptothecin/PLGA were 1:33:1 and 50: Empty microspheres (PLGA only) and 1:50 coumarin 6/PLGA microspheres were prepared using the method described above.

Physicochemical Characterization of Camptothecin/PLGA Microspheres  Particles were observed with an S-2250N scanning electron microscope (Hitachi, Tokyo, Japan) by coating samples with a 25-nm thick layer of gold using a model SC-701 quick carbon coater (Sanyo Electronics, Tokyo, Japan). The diameters of the microparticles (horizontal Feret’s diameters) were determined by image analysis from approximately 500 to 800 particles 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.

The camptothecin/PLGA microspheres were dispersed in dimethyl sulfoxide at a microsphere concentration of 1 mg/ml. The camptothecin concentration in the solution was measured by high-performance liquid chromatography (HPLC) (Class LC-10; Shimadzu, Kyoto, Japan); column, Mightysil RP-18 (4.6 mm × 250 mm, Shimadzu CBM 10A; auto-injector, Shimadzu SIL-10XL; column oven, phy (HPLC) (Class LC-10; Shimadzu, Kyoto, Japan) under the following conditions: pump, Shimadzu LC 10AT; detector, Shimadzu SPD-10A; module, Shimadzu CDM 10A; auto-injector, Shimadzu SIL-10XL; column oven, Shimadzu DGU-12A; column, Mityusil RP-18 (4.6 mm × 150 mm; Kanto Chemical, Tokyo, Japan); column temperature, 40 °C; detection wavelength, 254 nm; flow rate, 1.0 ml/min. The percent camptothecin encapsulation was determined as follows: % encapsulation = 100 × [measured camptothecin amount/theoretical camptothecin amount].

Camptothecin Release to Phosphate-buffered Saline (PBS) from Camptothecin/PLGA Microspheres Alone and from Camptothecin/PLGA Microspheres in TGP Gel  To measure the release of camptothecin from the camptothecin/PLGA microspheres to PBS, 1 mg camptothecin/PLGA microspheres were placed in a stoppered test tube containing 10 ml PBS. The test tube was mixed end-over-end using a revolution mixer (RVM-2048, Asahi Techno Glass, Tokyo, Japan) placed in a thermostatic oven at 37 °C. After 1, 3, 7, 14, 21, and 28 d, the mixture was centrifuged at 1560×g for 10 min. The resulting supernatant was collected, and the concentration of camptothecin was measured by HPLC.

TGP (freeze-dried product) was soaked in ultrapure water at 2% (w/v) in a glass test tube and stored at 4 °C. After the TGP absorbed the water, the tube was shaken occasionally for about 24 h to completely dissolve the TGP. The resulting TGP solution was sterilized by passing it through a 0.2-μm pore cellulose acetate membrane filter (Advantec Toyo, Tokyo, Japan) and then lyophilized. Sterilized and freeze-dried TGP was also dissolved in ultrapure water at 10% (w/v) and stored at 4 °C for 48 to 72 h to remove air bubbles. To measure the release of camptothecin from the camptothecin/PLGA microspheres suspended in TGP hydrogel, 10 mg camptothecin/PLGA microspheres were dispersed in 1 ml TGP solution. Next, 200 μl of the solution was placed on the membrane (0.48 cm²) of a chemotaxis culture chamber (ChemotaxcellTM, 3-μm pore size, Kurabo, Osaka, Japan). The chamber was placed in the opening part of a screw cap bottle containing 16 ml PBS and a magnetic stir bar, and the cap was screwed to close the bottle. The bottle containing the chamber was submerged in a 37 °C water bath equipped with magnetic stirrers (STW-300; As One, Osaka, Japan), and the solution in the bottle was stirred at 200 rpm. At selected time points, 1-ml samples of the test solution were removed, and replaced 1 ml PBS. The camptothecin concentration in the sample was measured by HPLC.

Cell Culture  Cells were grown in fetal bovine serum (FBS) (+) medium, consisting of Ham’s F-12 medium (Invitrogen, Carlsbad, CA, U.S.A.), pH 7.4 (adjusted with sodium bicarbonate) supplemented with 0.1% penicillin/streptomycin (10000 U/ml penicillin; 10000 μg/ml streptomycin; Invitrogen), 1 mM glutamine, and 10% FBS (Invitrogen). C6 rat glioma cells were seeded in 225 cm² tissue culture flasks with 10 ml FBS (+) medium and incubated in a humidified 5% CO₂ atmosphere at 37 °C for more than 24 h. The cells were washed three times with PBS and grown to 70—80% confluency in FBS (+) medium.

Rat Models of Glioma  Experiments were performed using male Sprague-Dawley rats (6 weeks old; 180—220 g; Japan SLC, Inc., Shizuoka, Japan). The animals had free access to food and water 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 Tokyo University of Pharmacy and Life Sciences. The rats were fasted for 1 d before the experiments. The rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg; Dainippon Sumitomo Pharma, Osaka, Japan) and their heads were fixed in position with a brain stereotaxic instrument for rats (SR-6R; Narishige, Tokyo, Japan). A part of the hair on the 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. The point at 3 mm to the left and 3 mm forward from Lambda of the bone of the skull was marked. A hole was drilled in the skull with a precision drill (Proxxon Mini Router No. 28511; Kiso Power Tool, Osaka, Japan) equipped with a 0.5 drill bit (Proxxon drill bit 0.5 mm No. 28854; Kiso Power Tool) at the marked point. A 30-gauge dental injection needle (Terumo, Tokyo, Japan) for injecting the brain was connected to one end of a 0.61-mm diameter polyethylene tube, and a 27-gauge needle (Terumo) connected to a 1-ml syringe (Terumo) was connected to the other end. The needle was subsequently passed through a microsyringe pump for animal experiments (CFV-2100; Nihon Kohden, Tokyo, Japan). The 30-gauge needle was inserted at a depth of 7 mm from the skull surface, and 10 μl C6 suspension (1×10⁶ cells) was injected at 3.33 μl/min. The 30-gauge needle was held in position for 5 min, and then withdrawn over a period of 3 min to prevent the cell suspension from flowing backwards. The hole in the skull was covered with bone wax (Lukens: Surgicel Specialties Corp., Reading, PA, U.S.A.), and the skin on the scalp skin was closed using a No. 4 suture needle (Natsume; Seiakusho, Tokyo, Japan) and sterilized with isodine (Meiji Seika Kaisha, Tokyo, Japan). Controls consisted of rats implanted with C6 cells
but without drug

Preparation of Histological Sections of Rat Brain. Reflux Fixation of Rats. Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg) 3, 7, 10, and 14 d after injection of TGP or 7, 14, and 21 d after stereotactic implantation of C6 cells. The abdominal hair was removed using an electric hair clipper. The abdominal rectus muscle was cut out along the midline of the abdomen with surgical scissors. The diaphragm then was cut out along with the costae, and the costae were cut while raising the breast bone to expose the heart. The right atrium of heart was then cut with ophthalmic scissors for exanguination. An injection needle (27 gauge) was inserted into the left atrium of the heart, and saline solution was circulated. When the eye color of the rats changed from red to clear and the blood flowing from the right atrium became clear, the reflux solution was changed to 4% paraformaldehyde. Reflux fixation was complete when the muscles around the mouth and jaw became rigid.

Removal and Fixation of Rat Brains. The scalp of reflux-fixed rats was cut along the midline of the head with surgical scissors, and the normal region was cut to expose the occipital area, the topmost cervical vertebra (C1), and the second cervical vertebra (C2). The spinal cord was cut between C1 and C2. Next, the head bone from the occipital bone to the frontal bone was cut along the midline of the skull, and the temporal bone was removed with tweezers as far as possible to expose the brain. The dura mater was removed, and the trigeminal, optic nerves, etc. were cut out to remove the brain. The brain was then fixed for 1 d in 4% paraformaldehyde.

Paraffin Embedding of Rat Brains and Preparation of Tissue Sections. The rat brains were washed with water for 6 h and immersed in 70% ethanol for 24 h. Paraffin infiltration and embedding of the brain were performed using a vacuum infiltration processor and tissue embedding console system (Tissue-Tek VIP 5; Sakura Finetek Japan, Tokyo, Japan). The brain was cooled to 4 °C to solidify the paraffin, and 20-μm paraffin-embedded sections were cut with a sledge microtome (NS-31; Yamato Koki, Saitama, Japan). The samples were dried at 37 °C for 24 h and then heated to 90 °C to melt the paraffin. Next, the sections were stained in hematoxylin and eosin. The tumor volume was determined as follows: tumor volume = longest diameter × shortest diameter² × 1/2.

Assessment of the Retention of F-TGP in the Brains of Rats. The brains of normal rats were treated with 30 μl of 10 w/v% of F-TGP (LCST was 20 °C) solution described for the injection of camptothecin/PLGA microparticles at 10 μl/min. The needle was kept in position for 5 min and then withdrawn over a period of 3 min to prevent the solutions from flowing backwards. The hole in the skull was sealed with bone wax, and the scalp skin was sutured and sterilized with iodine. On days 0, 7, 14, and 21, the brain was removed, and the tissue around the site of injection was excised. The tissue was illuminated with a UV lamp (Model UYG-54 Mineralight Lamp®; UVP, Upland, CA, U.S.A.), and the retention of the microspheres was assessed by observing the fluorescence from coumarin 6.

Statistical Analysis. Survival was evaluated using Kaplan–Meier survival curves. The mean survival period was defined as the day at which survival was 50%. Statistical analysis of the survival period was performed using a non-parametric log-rank Test.

Results and Discussion

Tumor Growth in Rat Glioma Models. In the hema-toxyl-stained slices of glioma from a rat brain 7, 14, and 21 d after stereotactic implantation of C6 cells, a tumor was hardly visible until 5 d after implantation of the cells (Fig. 3). After day 7, a strongly-stained area of the tumor was clearly visible in all samples. The tumor volume on days 7, 14, and 21 was 5.6, 31.6, and 140 mm³, respectively. The tumor occupied a large part of left side of the brain after day 21, and none of the rats survived thereafter. According to these observations, we decided to initiate treatment in the rats 7 d after the implantation of C6 cells.

Anti-tumor Effect of Camptothecin Solution in the Rat Glioma Model. As shown in Fig. 4, from the Kaplan–Meier survival curves of rats bearing intracranial C6 glioma tumors, the mean survival period was determined to be 15—16 d for the untreated group and one-time treated group at 7 d after tumor inoculation. A slight increase in the mean survival period was observed in the groups treated with the camptothecin solution at 7, 10, and 13 d after tumor inoculation (19 d), suggesting that continuous supply of camptothecin to the brain tumor is effective for survival.

Scanning Electron Microscopy (SEM) Images of Camptothecin/PLGA Microparticles and Camptothecin Release Profiles. SEM analysis of the camptothecin/PLGA microspheres at various ratios of camptothecin to PLGA revealed all microspheres with spherical shapes. The mean diameters at camptothecin/PLGA ratios of 1 : 33 and 1 : 50 were 32 and 39 μm (Fig. 5). The encapsulation efficiencies at camptothecin/PLGA ratios of 1 : 33 and 1 : 50 were 34±8% and 37±9%, respectively.

The camptothecin release from camptothecin/PLGA microspheres to PBS and through the TGP gel to PBS were analyzed (Fig. 6). Sustained and long-term release of camptothecin was observed at 1 : 33 and 1 : 50 camptothecin/PLGA ratios. Although the camptothecin release through the TGP gel to PBS was slightly slower than directly to PBS, the release profiles were very similar. Thus, camptothecin release from camptothecin/PLGA microspheres promptly disperses in the TGP gel with hydrolysis of PLGA and diffuses to PBS, with the release from PLGA being the rate-limiting step.

Residual Performance of F-TGP in Rat Brains after
Administration of F-TGP Solution
Analysis of rat brain cross-sections after F-TGP administration on days 0, 7, and 28 revealed fluorescence after day 28 (Fig. 7). The residual percentages of F-TGP on days 0, 7, and 28 were 100%, 91.4%, and 83.0%, respectively (n=5), indicating that F-TGP was retained in the rat brains for a long time after injection. Although LCST is different between F-TGP (20°C) and TGP (32°C), the TGP solution injected in the rat brains changed to a gel, resulting in long-term retention of TGP.

Effect of TGP on the Brain Tissue after Administration of TGP Solution
The biocompatibility, complete pathogen-free property, and cytotoxicity of TGP have already been reported by Shimizu et al.38 We studied the effect of TGP on brain tissue after injection of TGP solution to the left side of the brain by hematoxylin staining (Fig. 8). Stained sections of rat brains 3, 7, 10, and 14 d after administration detected no TGP in the brain during the preparation process of the samples. Inflammation of brain tissue based on the injection of the needle was observed until day 10 after administration, but was undetected after day 14. From the comparison of the right and left sides of brain, no deformation and damage of brain tissue from TGP were observed, suggesting that the effect of TGP on brain tissue is small.

Anti-tumor Effect of Camptothecin/PLGA Microspheres in TGP in the Rat Glioma Model
At room temperature, 10% (w/v) of TGP is in the sol state, allowing for dispersal of the camptothecin/PLGA microspheres. This suspension was injected into the brain of rat glioma models, and TGP, which is in the gel state at around 37°C, is expected to convert back into the gel state in the brain to retain the camptothecin/PLGA microspheres around the injection site. Figure 9 shows the Kaplan–Meier survival curves of rats bearing intracranial C6 glioma tumors treated with TGP-only solution, camptothecin powder dispersed in TGP solution, empty PLGA microspheres dispersed in TGP solution, and camptothecin/PLGA microspheres dispersed in PBS or TGP solution 7 d after tumor inoculation; the camptothecin dose was 1 mg. The mean survival period of the untreated group was 16 d, with all rats dying by day 19 after tumor inoculation. The mean survival period of rats treated with TGP-only solution and empty microspheres in the TGP group was not significantly longer than the controls (18 d). The mean survival period of rats treated with 1 : 50 camptothecin/PLGA in PBS was slightly increased at 19 d, while that of rats treated with the camptothecin powder dispersed in TGP solution was 21 d, with all rats dying by day 23 after tumor inoculation. In contrast, the mean survival periods of rats treated with camptothecin/PLGA microspheres in TGP solution were significantly longer than the control group. In rats treated with 1 : 33 or 1 : 50 camptothecin/PLGA in TGP solution, which showed long-term and sustained release of camptothecin, the mean survival time clearly increased. Furthermore, some rats...
survived for long periods when treated with 1:33 and 1:50 camptothecin/PLGA in TGP solution. Specifically, one of the four rats treated with 1:33 camptothecin/PLGA in TGP solution survived more than 30 d, and two of the six rats treated with 1:50 camptothecin/PLGA in TGP solution survived more than 60 d.

When the coumarin 6/PLGA microspheres were dispersed in PBS, fluorescence of coumarin 6 in the microspheres was observed on day 7 after administration and decreased until day 14 (Fig. 10). When the coumarin 6/PLGA microspheres were dispersed in TGP solution, the fluorescence was clearly observed 7 d after injection, and it was still observed on day 14, indicating that TGP was able to prolong the retention of microspheres at the injection site. This retention at the injection site by TGP in the gel state resulted in sustained and long-term release from the microspheres and thus a continuous and long-term delivery of camptothecin to the glioma cells. This action in turn corresponded to an increased therapeutic efficiency of the camptothecin/PLGA microspheres.

**Conclusion**

In the current study, we prepared sustained release camptothecin/PLGA microspheres and dispersed them in TGP solution. This suspension was injected into the brains of rats implanted with C6 glioma cells. The camptothecin/PLGA microspheres remained at the tumor site due to the gel state of TGP, thus resulting in continuous, long-term release of camptothecin and an increased anti-tumor effect.

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