Convection-Enhanced Delivery of a Synthetic Retinoid Am80, Loaded into Polymeric Micelles, Prolongs the Survival of Rats Bearing Intracranial Glioblastoma Xenografts

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Prognosis for the patients with glioblastoma, the most common malignant brain tumor, remains dismal. A major barrier to progress in treatment of glioblastoma is the relative inaccessibility of tumors to chemotherapeutic agents. Convection-enhanced delivery (CED) is a direct intracranial drug infusion technique to deliver chemotherapeutic agents to the central nervous system, circumventing the blood-brain barrier and reducing systemic side effects. CED can provide wider distribution of infused agents compared to simple diffusion. We have reported that CED of a polymeric micelle carrier system could yield a clinically relevant distribution of encapsulated agents in the rat brain. Our aim was to evaluate the efficacy of CED of polymeric micellar Am80, a synthetic agonist with high affinity to nuclear retinoic acid receptor, in a rat model of glioblastoma xenografts. We also used systemic administration of temozolomide, a DNA-alkylating agent, which has been established as the standard of care for newly diagnosed malignant glioma. U87MG human glioma cells were injected into the cerebral hemisphere of nude rats. Rats bearing U87MG xenografts were treated with CED of micellar Am80 (2.4 mg/m²) on day 7 after tumor implantation. Temozolomide (200 mg/m²/day) was intraperitoneally administered daily for 5 days, starting on day 7 after tumor implantation. CED of micellar Am80 provided significantly longer survival than the control. The combination of CED of micellar Am80 and systemic administration of temozolomide provided significantly longer survival than single treatment. In conclusion, temozolomide combined with CED of micellar Am80 may be a promising method for the treatment of malignant gliomas.

Keywords: Am80; glioblastoma; convection-enhanced delivery; temozolomide; polymeric micelle

Glioblastoma is the most common primary malignant brain tumor in adults. Despite therapeutic advances, the median survival continues to be approximately 12 months. Therefore, a new therapeutic approach is required.

Convection-enhanced delivery (CED) is a relatively new method that might overcome the problems posed by the requirements of local drug delivery (Bobo et al. 1994). CED uses a pressure gradient established at the tip of an infusion catheter to create bulk-flow that pushes the drug through the interstitial spaces. CED of therapeutic agents bypasses the blood-brain barrier, delivers high concentrations of therapeutic agents to the target site, and minimizes systemic exposure, thus resulting in fewer side effects.

CED has been used to deliver many antineoplastic agents in animal studies with promising outcomes (Bruce et al. 2000; Degen et al. 2003; Vogelbaum 2007). However, CED of free drugs has various problems including rapid clearance from the tumor interstitium, so no selective accumulation in the targeted tissue can be achieved (Kunwar et al. 2007). We considered that it was necessary to develop a new pharmaceutical composition comprising an effective CED agent.

Drug carrier systems offer the advantage of sustained drug release as well as targeting of specific sites. Liposomes have been used as drug carriers in combination with CED (Saito et al. 2004, 2006a, 2006b; Noble et al. 2006; Yamashita et al. 2007; Kikuchi et al. 2008). Recently, we demonstrated the therapeutic efficacy of a newer type of drug carrier system, polymeric micellar doxorubicin, which was infused by CED in rat brain tumor models (Inoue et al. 2009). Polymeric micelles are an assembly of synthetic polymers, which typically block copolymers with both hydrophobic and hydrophilic properties. Polymeric micelle
carrier systems were first studied for targeting solid tumors by intravenous injection (Yokoyama et al. 1990, 1999). Polymeric micelle carrier systems are electrically neutral and have the so-called stealth property that evades rapid clearance by the reticuloendothelial system. Consequently, systemic administration of polymeric micelle systems is effective against solid tumors because of the enhanced permeability and retention effect, which depends on the hyper-permeable vasculature and the absence of effective lymphatic drainage that prevents efficient clearance of macromolecules in the solid tumor tissues (Greish 2007). Various micelle-encapsulated cytotoxic agents are currently undergoing clinical evaluation for systemic administration, including doxorubicin (Tsukioka et al. 2002), paclitaxel (Hamaguchi et al. 2007), cisplatin (Uchino et al. 2005), and a camptothecin derivative SN-38 (Koizumi et al. 2006).

All-trans retinoic acid (ATRA) and other retinoids are reported to inhibit the growth rate of various malignancies including acute polymyelocytic leukemia, lung cancer, and glioblastoma both in vivo and in vitro (Flynn et al. 1983; Yung et al. 1996; Jaeckle et al. 2003). ATRA induces cell differentiation, cell cycle arrest, apoptotic cell death, and inter-leukin 6/inter-leukin 6 receptor downregulation in vitro. Retinoid effects are mediated through the interaction with two types of nuclear receptors, retinoic acid receptor (RAR) and retinoid X receptor (RXR), each of which has three subtypes (α, β, and γ). ATRA is one of the most clinically effective retinoids; nevertheless, high rates of adverse effects have been reported such as exanthesis, fever, and xeroderma (Delva et al. 1993). The adverse effect on the skin is due to the large number of RAR-γ receptors distributed in the skin. The definition of retinoids has been expanded to include molecules that bind to RARs and RXRs, regardless of the similarity in molecular structure to ATRA. Am80 is such a synthetic retinoid with strong binding affinity to the nuclear receptors, but has a very different chemical structure to ATRA (Tobita et al. 1997). Am80 is a RAR-αβ-selective retinoid that does not activate RAR-γ and RXRs, so it may not cause adverse effects. Am80 is a promising candidate for CED infusion because glioma cells have extensive expression of RAR-αβ (Chattopadhyay et al. 2001; Costa et al. 2001). Alkylating agents and retinoids are among the chemotherapeutic agents that have shown activity against gliomas, either individually or in combination. Temozolomide and 13-cis-retinoic acid have also shown activity against recurrent gliomas in phase II clinical trials (Yung et al. 1996; Wismeth et al. 2004).

In the present study, we evaluated the efficacy of CED administration of micellar Am80 and/or systemic administration of temozolomide in the intracranial xenograft model.

**Methods**

**Preparation of Agents**

Am80 was kindly provided by Dr. Koichi Shudo of the Research Foundation Itsuu Laboratory (Tokyo, Japan). Temozolomide was provided by Schering-Plough K.K. (Osaka, Japan) and was dissolved in a solution of 0.1% dimethyl sulfoxide (Sigma Chemical Co., St. Louis, MO) in 0.9% NaCl solution.

Am80 was incorporated into a polymeric micelle formed from poly(ethylene glycol)-β-poly(benzyl aspartate) block copolymer. The polymer synthesis, Am80 incorporation into the polymeric micelle carrier, and characterization of the carrier system were as previously described (Satoh et al. 2009). Briefly, the block copolymer and Am80 were dissolved in tetrahydrofuran, and the obtained solution was subjected to solvent evaporation. Water was added to the dried residue, followed by sonication. The micelle solution was centrifuged to remove any insoluble precipitate (3,900 rpm, 10 min, 20°C) and then filtered through a Millex 0.22 μm PVDF filter (Millipore Corp, Billerica, MA). The composition of the block copolymer was as follows. The mean molecular weight of the poly(ethylene glycol) chain was 5,200, and the mean unit number of the poly(aspartic acid) chain was 24. Hydrophobic benzyl ester was formed at 83 mol% of the aspartic acid residue. Therefore, the mean molecular weight of the poly(aspartate) chain (83% benzyl aspartate residues and 17% aspartic acid residues) was 4600. In addition, N,N-dimethyloctadecylamine was added as a hydrophobic amine with Am80 at a molar ratio of 1:1 in the micelle preparation step. The drug content by weight in the polymeric micelle was 14%.

**Tumor Cell Line**

The established human glioblastoma cell line U87MG was obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained as monolayers in a complete medium consisting of Eagle’s minimal essential medium supplemented with 10% fetal calf serum, non-essential amino acids, and 100 U/ml penicillin G. Cells were cultured at 37°C in a humidified atmosphere consisting of 95% air and 5% CO₂.

**Cell Viability Assay**

The cell viability of U87MG cell lines treated with Am80 and temozolomide was assessed using the MTS assay (CellTiter96 Aqueous One Solution Cell Proliferation Assay; Promega Corp, Madison, WI). MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), was reduced by living cells in the presence of phenazine methosulfate (PMS) to yield a purple formazan product that could be assayed colorimetrically. Cells were seeded at 750 cells per well in 75 µl of medium in 96-well flat-bottom plates and grown overnight at 37°C in an incubator. Temozolomide was used at 100 µM as described previously (Das et al. 2005). After exposure to Am80 (0, 50, 100, 200, or 500 µM), temozolomide (100 µM), and a combination of the two agents (100 µM Am80 + 100 µM temozolomide) for 24 hours, the plates were assayed with a microplate reader (Softmax Pro; Molecular Devices Corp, Sunnyvale, CA). Results were compared using one-way analysis of variance (ANOVA) with Tukey’s Multiple Comparison test at a 95% confidence interval.

**Western Blot Analysis**

On the day before treatment, 1 × 10⁶ cells per dish were seeded. After 24 hours incubation, cells were treated with Am80 (100 µM), temozolomide (100 µM), or a combination of both agents. Cells were collected 24 hours and 48 hours after the treatment. Protein was extracted from these cells with a mammalian protein extraction reagent (M-PER; Thermo Scientific, Rochester, NY). Samples were then prepared in sample buffer (Novex; Invitrogen, Carlsbad, CA) and
heated to 94°C for 5 minutes. Samples were then subjected to electrophoresis on 10% polyacrylamide gels (or 16% gels only for cleaved caspase-3), and then blotted onto polyvinylidene fluoride membranes (PDVF) (Invitrogen). PDVF were then incubated overnight with primary antibody against phospho-mitogen-activated protein kinase (phospho-MAPK) (Cell Signaling Technology, Cambridge, MA; 1:200), phospho-Akt (Cell Signaling Technology; 1:200), cleaved caspase-3 (Cell Signaling Technology; 1:500), cleaved caspase-9 (Cell Signaling Technology; 1:500), and β-actin (Santa Cruz Technology, Santa Cruz, CA; 1:1000). The residue targets for each phospho-specific antibody were p-MAPK (Thr202/Tyr204) and p-Akt (Thr308).

Animals

Male Sprague–Dawley rats weighing approximately 200 g were obtained from Charles-River Japan, Inc. (Yokohama, Kanagawa, Japan). Seven-week-old male Fischer 344/Nijc1-μm/μm (nude) rats were purchased from CLEA Japan, Inc. (Tokyo, Japan). All protocols used in the animal studies were approved by the Institute for Animal Experimentation of Tohoku University Graduate School of Medicine.

Intracranial Tumor Implantation

U87MG cells were harvested by trypsinization, washed once with Hank’s balanced salt solution without Ca\(^{2+}\) and Mg\(^{2+}\) (HBSS), and resuspended in HBSS for implantation. A cell suspension containing 5 × 10^5 cells per 10 μl of HBSS was used for implantation into the striatum of rat brains. Rats were placed in a small animal stereotactic frame (Narishige Manufacturing Co., Ltd., Japan) under deep halothane anesthesia. A sagittal incision was made through the skin to expose the cranium, and a burr hole was made in the skull at 0.5 mm anterior and 3 mm lateral from the bregma using a small dental drill. Cell suspension (5 μl) was injected at a depth of 4.5 mm from the brain surface. After a wait of 2 minutes, another 5 μl was injected at a depth of 4 mm. After a final wait of 2 minutes, the needle was removed, and the wound was closed with sutures.

CED

CED of micellar Am80, free Am80, or PBS was performed with a volume of 20 μl as described previously (Saito et al. 2006b). The infusion system consisted of a reflux free step-design infusion cannula connected to a loading line (containing micellar Am80, free Am80, or PBS) and an olive oil infusion line. A 1-ml syringe (filled with olive oil) mounted onto a micro-infusion pump (BeeHive; Bioanalytical Systems, West Lafayette, IN) regulated the flow of fluid through the system. On the basis of the chosen coordinates, the infusion cannula was mounted onto stereotactic holders and guided to the target region of the brain through the same burr holes made in the skull at tumor implantation. The following ascending infusion rates were applied for the 20-μl infusion: 0.2 μl/min for 15 minutes, 0.5 μl/min for 10 minutes, and 0.8 μl/min for 15 minutes.

Evaluation of Micellar Am80 Toxicity

Sprague–Dawley rats (3 rats in each group) received a single 20 μl CED infusion of micellar Am80. To ensure the safety of micellar Am80, the starting dose of 2.4 mg/m^2, available highest dose of micellar Am80, was chosen. The dose of 1.2 mg/m^2 and 0.6 mg/m^2 were also evaluated. Rats were monitored daily for survival, weekly for weight, and for general health (alertness, grooming, feeding, excreta, skin, fur, mucous membrane conditions, ambulation, breathing, and posture). The rats were euthanized 6 weeks after the CED treatment, and their brains were removed, fixed, sectioned (5 μm), and stained with hematoxylin and eosin and examined using a stereoscopic microscope (SZX7; Olympus Corp, Tokyo, Japan) and a light microscope (ECLIPSE 80i; Nikon Corp, Tokyo, Japan).

Survival Studies

Thirty rats implanted with U87MG tumor cells were randomly assigned to 6 groups of 5 rats: 1) a control group treated with CED of PBS (20 μl solution), 2) a group that underwent systemic treatment with temozolomide (200 mg/m^2/day), 3) a group treated with CED of free Am80 (2.4 mg/m^2 Am80 in a 20 μl solution), 4) a group treated with CED of micellar Am80 (2.4 mg/m^2 Am80 in a 20 μl solution), 5) a group that underwent systemic treatment with micellar Am80 (2.4 mg/m^2 Am80 in a 20 μl solution) and systemic treatment with temozolomide (200 mg/m^2/day), and 6) a group treated with CED of Am80 (2.4 mg/m^2 Am80 in a 20 μl solution) and systemic treatment with temozolomide (200 mg/m^2/day). CED infusion of free Am80 or micellar Am80 was performed on day 7 after tumor implantation. Systemic treatment with temozolomide consisted of a dose of 200 mg/m^2/day in a solution of 10% dimethyl sulfoxide in 0.9% NaCl solution for a total volume of 90 ml/m^2, which was intraperitoneally administered daily for 5 days, starting on day 7 after tumor implantation. Systemic treatment of micellar Am80 was performed by injection through the tail vein 7 days after tumor implantation. Rats were monitored daily for survival and general health. Survival rates in the treatment groups were compared using a log-rank test. Estimated survival was expressed as a Kaplan-Meier curve.

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Staining

Eight rats implanted with U87MG tumor cells were randomly assigned to 4 groups of 2 rats: 1) a control group, 2) a group treated with CED of micellar Am80, 3) a group treated with systemic temozolomide, and 4) a group treated with CED of micellar Am80 and systemic temozolomide. Paraffin sections made from the brains of 2 rats from each group euthanized 12 days after tumor implantation were examined for apoptosis. The sections were deparaffinized followed by incubation with NaN\(_3\) and H\(_2\)O\(_2\) in PBS with 0.3% Triton-X for 20 minutes at room temperature. Then, the slides were washed 3 times with PBS. After incubation with terminal deoxynucleotidyl transferase (TdT) buffer (TdT, Recombinant; Invitrogen) for 15 minutes at room temperature, a mixture of TdT (2.5 μl) (Invitrogen), biotinylated 16-dUTP (6 μl) (Roche Diagnostics, Mannheim, Germany), and TdT buffer (100 μl) was added to each slide for 60 minutes at 37°C. Then, the slides were washed 2 times with TB buffer (6 mM sodium citrate, 60 mM NaCl) and blocked by incubation in 2% bovine serum albumin in PBS for 15 minutes at room temperature, followed by washing 3 times after blocking and incubation in an avidin-biotin peroxidase complex (ABC) solution (VECTASTAIN Elite ABC Standard Kit; Vector Laboratories Inc., Burlingame, CA) in PBS for 30 minutes at room temperature. The slides were twice washed with 0.175 M sodium acetate for 10 minutes and then reacted with 3’3-diaminobenzidine hydrochloride for appropriate times. Counterstaining was performed with methyl green solution.
Results

Enhanced Cytotoxic Effects of Am80 and Temozolomide In Vitro

Am80 significantly reduced the viability of U87MG cells in a dose-dependent manner through U87MG cells seeded in a 96-well flat-bottom plate and treated after 24 hours, then incubated for 2, 4, and 6 days (Fig. 1A). Inhibition of cell growth was high at 4 and 6 days after treatment. The 50% inhibition concentration (IC50) of Am80 was 123 µM. In combination with temozolomide, Am80 was used at 100 µM, approximate IC50 concentrations. Am80 combined with temozolomide achieved additional reduction in cell viability (Fig. 1B). Expression levels of phospho-Akt, phospho-MAPK, cleaved caspase-3, cleaved caspase-9, and β-actin were evaluated by western blot analysis using antibodies that detect only the phosphorylated forms of these proteins (Fig. 2). Treatment with Am80 or temozolomide showed a modest decrease in the expression of phospho-Akt protein, but treatment with Am80 and temozolomide further decreased the expression. Expression of phospho-MAPK was strongly suppressed in cells treated with Am80 or temozolomide, and the suppression effect was sustained in cells treated with these in combination. Western blot analyses for cleaved caspase-3 and cleaved caspase-9 indicated the activation of caspase-3 and caspase-9, respectively, in U87MG cells treated with temozolomide, Am80, or the combination of temozolomide and Am80. Similar levels of activation of both caspases were observed in the U87MG cells treated with temozolomide alone and Am80 alone. The combination of Am80 and temozolomide enhanced activation of both caspases. The molecular events occurring during synergistic induction of cell death due to temozolomide and Am80 were characterized in U87MG cells.

Toxicity of Am80 in Normal Brain Parenchyma

No dose-limiting toxicity was found at 0.6, 1.2, or 2.4 mg/m². Animals that received CED of micellar Am80 at 2.4 mg/m² or less showed evidence of minor tissue damage at the site of the infusion cannula in the striatum, but no other apparent toxicity (Fig. 3).

Efficacy of Combined Micellar Am80 and Temozolomide in U87MG Brain Tumor Xenograft In Vivo

All rats from the control group had to be euthanized because of tumor progression between 12 to 16 days after implantation. Single treatment with CED of free Am80 showed no improvement in survival. In contrast, CED of micellar Am80 provided significantly longer survival \((p = 0.019, \text{log-rank test})\). Furthermore, the combination treatment of CED of micellar Am80 and systemic treatment of temozolomide provided significantly longer survival than the single treatment \((p = 0.0027 \text{ compared with the CED of micellar Am80 group})\). However, the combination of systemic administration of micellar Am80 and temozolomide did not achieve longer survival than systemic temozolomide alone (Fig. 4).

Detection of Apoptotic Cell Death after Treatment

TUNEL staining was performed on brain slices of rats from each group euthanized 6 days after treatment had started. Tumors from rats treated with CED of micellar Am80 and temozolomide exhibited decreased tumor density and increased number of TUNEL-positive cells compared with those from animals treated with only agent or control animals (Fig. 5).

Discussion

In the present study, we found that the CED of micellar Am80 provided long survival for rats bearing U87MG xenografts. We previously reported that micellar agents infused by CED were extensively distributed in normal rat
Efficacy of Micellar Am80 with Systemic Temozolomide

We also reported that micellar agents were distributed over almost the entire tumor area, including tumor margins, in rat brain tumor models (Inoue et al. 2009). The distribution of agents infused by CED in the rat brain is significantly increased if the infusate is more hydrophilic, which implies less tissue affinity (Saito et al. 2004, 2006b; MacKay et al. 2005; Yamashita et al. 2007). Furthermore, polyethylene glycol encapsulation provides steric stabilization, reduces surface charge, and achieves better distribution (Inoue et al. 2009). In the present study, the poorer brain/tumor tissue distribution caused by Am80 hydrophobicity might be overcome by polymeric micelle carrier system.

On the other hand, micellar Am80 administered intravenously did not contribute to long survival for rats bearing U87MG xenografts, despite the fact that micellar agents have been shown to accumulate around tumor vessels and effectively pass through brain tumor vessels (Kuroda et al. 2009). Although this study lacks monitoring or confirmation of infusate, it is speculated that micellar Am80 administered systemically could not sufficiently penetrate into the hypovascular central area of implanted tumor with three-dimensional cellular structures, in which the diffusion of infusate was restricted. Development of monitoring of the distribution of infusate is required to gain a deeper appreciation of micellar Am80.

Several chemotherapeutic agents delivered locally using CED, including 1,3-bis(chloroethyl)-1-nitrosourea (Bruce et al. 2000), gemcitabine, and carboplatin (Degen et al. 2003), prolonged survival of rats bearing U87MG xeno-

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Fig. 2. Western blotting analysis for phospho-Akt, phospho-MAPK, cleaved caspase-3 and -9, and β-actin. The data shown are representative of three experiments.

Fig. 3. Toxicity evaluation of micellar Am80 after CED infusion into the brain striatum of wild-type rats. There was only a slight cannulation scar; an arrow indicates small fibrous tissues with inflammation. No damage was induced to the infused hemisphere in any rats. Hematoxylin and eosin stain, original magnification ×8 in a stereoscopic microscope (A) and ×200 in a light microscope (B).
grafts. However, some of these agents applied locally to the cerebrospinal fluid have long-term side effects including leukoencephalopathy and brain atrophy (Shapiro and Young 1984). Ideally, agents for CED administration into brain tumors would show the highest possible therapeutic ratio against tumor cells over normal cells. Retinoids such as Am80 show selective toxicity against neoplastic transformed cells, so they represent an excellent candidate for local delivery (Schneider et al. 2000). In our study, the toxicity of Am80 against normal brain cells was minimal, even with enhanced delivery. These results suggest that CED-delivered Am80 may be a viable therapeutic option (Chattopadhyay et al. 2001).

The most important function of the carrier is the inhibition of rapid drug absorption by cells at the injection site, since the agent cannot be distributed over a large volume of tissue in the presence of rapid absorption. Sustained drug release is needed for effective inhibition of rapid drug absorption by cells. In this study, we prepared polymeric micelles containing Am80, which were observed to sustain
the release of Am80 in vitro more than that of free Am80 (Satoh et al. 2009). For this reason, CED of this micelle was more effective than that of the free agent.

We chose temozolomide as a drug in combination with Am80 because the use of temozolomide is already clinically established for glioma therapy. The strategy for induction of differentiation followed by activation of apoptosis has been highly promising in cancer therapy. We found that Am80 and/or temozolomide treatment down-regulated cell growth signaling, p-MAPK. Activation of Akt is a major event in the development of glioblastoma, and high levels of Akt were frequently expressed in glioblastoma (Sonoda et al. 2001; Choe et al. 2003). Phosphorylation of Akt results in activation of Akt kinase activity, which has the potential to deregulate the cell cycle and suppress apoptosis. The present study demonstrated that treatment of U87MG xenografts with Am80 and temozolomide down-regulated phospho-Akt and activated cleaved caspase-3 and -9, indicating activation of the caspase cascade for apoptosis. In the survival study, CED of micellar Am80 individually or in combination with temozolomide improved the therapeutic outcome in the human glioblastoma model.

Agents such as temozolomide combined with CED administration of micellar Am80 may be promising for the treatment of malignant gliomas.

References


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