Rhenium-186 liposomes as convection-enhanced nanoparticle brachytherapy for treatment of glioblastoma

William T. Phillips, Beth Goins, Ande Bao, Daniel Vargas, Juan E. Gutierrez, Abram Trevino, Jessica R. Miller, James Henry, Richard Zuniga, Giacomo Vecil, and Andrew J. Brenner

Cancer Therapy and Research Center (A.J.B., A.T., J.R.M., R.Z.); Department of Radiology (B.G., A.B., D.V., J.E.G., W.T.P.); Department of Pathology (J.H.); and Department of Neurosurgery (G.V.), The University of Texas Health Science Center at San Antonio, San Antonio, Texas

Although external beam radiation is an essential component to the current standard treatment of primary brain tumors, its application is limited by toxicity at doses more than 80 Gy. Recent studies have suggested that brachytherapy with liposomally encapsulated radionuclides may be of benefit, and we have reported methods to markedly increase the specific activity of rhenium-186 (^{186}Re)–liposomes. To better characterize the potential delivery, toxicity, and efficacy of the highly specific activity of ^{186}Re -liposomes, we evaluated their intracranial application by convection-enhanced delivery in an orthotopic U87 glioma rat model. After establishing an optimal volume of 25 μL , we observed focal activity confined to the site of injection over a 96-hour period. Doses of up to 1850 Gy were administered without overt clinical or microscopic evidence of toxicity. Animals treated with ^{186}Re -liposomes had a median survival of 126 days (95% confidence interval [CI], 78.4–173 days), compared with 49 days (95% CI, 44–53 days) for controls. Log-rank analysis between these 2 groups was highly significant ($P = .0013$) and was even higher when 100 Gy was used as a cutoff ($P < .0001$). Noninvasive luciferase imaging as a surrogate for tumor volume showed a statistically significant separation in bioluminescence by 11 days after 100 Gy or less treatment between the experimental group and the control animals ($\chi^2[1, N = 19] = 4.8$; $P = .029$). MRI also supported this difference in tumor size. Duplication of tumor volume differences and survival benefit was possible in a more invasive U251 orthotopic model, with clear

separation in bioluminescence at 6 days after treatment ($\chi^2[1, N = 9] = 4.7$; $P = .029$); median survival in treated animals was not reached at 120 days because lack of mortality, and log-rank analysis of survival was highly significant ($P = .0057$). Analysis of tumors by histology revealed minimal areas of necrosis and gliosis. These results support the potential efficacy of the highly specific activity of brachytherapy by ^{186}Re -liposomes convection-enhanced delivery in glioma.

Keywords: brachytherapy, glioblastoma, glioma, liposomes, nanoparticles.

Glioblastoma (GBM) is the most common and most aggressive of the primary malignant brain tumors in adults. Annually, there are approximately 13,000 cases of GBM diagnosed, with historical 1-year and 5-year survival rates of 29.3% and 3.3%, respectively.¹ The poor survival is attributable partly to the infiltrative nature of the tumor, which makes it difficult to eliminate microscopic disease despite macroscopic gross-total resection. After combined modality therapy, 90% of patients have recurrence at the original tumor location.² The location of the tumor also makes drug delivery difficult, with only small or lipophilic molecules able to cross the blood-brain barrier to reach the tumor. The most significant advancement in treatment of GBM over the past several years has come from concomitant chemoradiotherapy with temozolomide, which can result in increased median survival of 14.6 months with radiotherapy plus temozolomide, compared with 12.1 months with radiotherapy alone.³ However, this still leaves much to be desired, with an improvement in median survival of only 2.5 months over radiotherapy alone.

Received March 17, 2011; accepted January 6, 2012.

Corresponding Author: Andrew J. Brenner, Mail Code 8232, Cancer Therapy and Research Center, 7979 Wurzbach Road, San Antonio, TX 78229 (brennera@uthscsa.edu).

Because most recurrences occur within 2 cm of the resection margin,⁴ local therapies for GBM that are delivered by convection-enhanced delivery (CED) are currently under investigation.^{5,6} Recent CED research has focused in part on lipid nanoparticles (liposomes) as carriers of anticancer agents for treatment of brain tumors.⁷⁻⁹ When administered by CED, liposomes appear to enhance the spread and the retention of the therapeutic agents throughout the tumor, compared with unencapsulated small molecules alone.^{10,11} Beta-emitting radionuclides encapsulated in nanoparticles appear to have significant advantages for brachytherapy, because the 2–4-mm path lengths of the beta particles (100–200 cell diameters) provide a micro-field of therapy within the tumor. This micro-field decreases the requirement of rigorous intratumoral distribution homogeneity required for traditional chemotherapeutic drugs in which the drug has to reach and enter every cancer cell for total tumor eradication. Recently, others have reported the CED administration of solid lipid nanoparticles carrying therapeutic beta-emitting radionuclides in a rat glioma model with promising results.¹² However, the specific activity achieved was relatively low, resulting in a maximum dose of only 12 Gy. Despite this low level of activity, some improved survival was achieved.

To make it possible to use liposomes carrying beta-emitting therapeutic radionuclides for glioma therapy, a method of conveniently loading therapeutic radionuclides into lipid nanoparticles with great efficiency and stability is required. Recently, we have successfully developed a method of loading gamma-emitting technetium-99m (^{99m}Tc) and 2 different energy beta-emitting radionuclides, ¹⁸⁶Re and ¹⁸⁸Re, into liposomes with high efficiency and specific activity.¹³⁻¹⁶ We have also shown very promising results with rhenium-liposomes for the treatment of rat models of head and neck cancer^{11,15} and intraperitoneally disseminated ovarian cancer metastasis.¹⁷ This method of loading high amounts of beta-emitting rhenium radionuclides into liposomes has been successfully reproduced in other laboratories and shown to be effective for cancer therapy.¹⁸⁻²⁰ This labeling method results in a markedly higher level of specific activity than has been previously described with solid rhenium-labeled nanoparticles,¹² providing the potential to deliver markedly higher therapeutic doses to tumors with minimally increased toxicity. In this study, we assessed the potential of ¹⁸⁶Re-liposomes produced with high specific activity for the treatment of GBM with use of an intratumoral application by CED in orthotopic U87 and U251 glioma rat models.

Materials and Methods

Xenografts

Human glioma cell line U-87 MG-luc2 expressing firefly luciferase was purchased from Caliper LRS. Cells were grown as described in product insert. The U251 glioma line expressing firefly luciferase was

generously provided by Luiz Penalva at the Children Cancer Research Institute at The University of Texas Health Science Center at San Antonio. Athymic HsdHan:RNU-Foxn1^{tmu} nude rats were purchased from Harlan Laboratories. Animals were anesthetized using isoflurane continuous inhalation (3% in 100% oxygen) and placed into a Stereotaxic Instrument (Stoelting). The scalp was prepped aseptically, a 0.5–1.5 cm longitudinal incision was made with an approximate scalpel, the skin was retracted laterally, and the bregma was identified. The injection site was localized by mapping the distance from the bregma using 1 mm forward and 4mm lateral. A hole was created in the skull at the site of injection with use of a small manual hand drill, and a 2.6-mm guide screw with a central 0.5-mm diameter hole was placed;²¹ 1×10^6 cells suspended in 5 μ L of saline were injected at a depth of 4.5 mm through the guide screw using a 10 μ L Hamilton syringe with a 27-gauge needle over 5 min. Tumor growth was observed using intraperitoneal injection of 250 μ L of 57 μ g/ μ L luciferin with in vivo imaging using an IVIS Lumina system and quantification by Living Image software (Caliper LRS).

Liposome Preparation

Liposomes of 100-nm diameter possessing both ammonium sulfate pH and glutathione (GSH) gradients were prepared and characterized using a modified protocol described previously.^{15,16} The liposomes were composed of distearoylphosphatidylcholine (DSPC; Avanti Polar Lipids) and cholesterol (Calbiochem) (molar ratio 55:45). Lipid mixture was dissolved with chloroform and then dried by rotary evaporation and overnight desiccation to form a lipid film. The dried lipid film was rehydrated with 300 mM sucrose in sterile water at 120 mM total lipid concentration and warmed to 55°C, followed by an overnight lyophilization. The dried lipid-sucrose mixture was rehydrated with 200 mM GSH (Sigma) and 300 mM ammonium sulfate (Sigma; pH, 5.1) in sterile water at 60 mM total lipid concentration, then subjected to 5 freeze-thaw cycles, followed by sequential extrusion through polycarbonate filters with different pore sizes (2 μ m, 1 μ m, 400 nm, and 200 nm, 2 passes each; and 100 nm, 5 passes) at 55°C (Lipex Extruder, Northern Lipids). After extrusion, liposomes were repeatedly ultracentrifuged at 41 000 rpm (Ti 50.2 rotor; Beckman) for 50 min and washed with 300 mM ammonium sulfate containing 75 mM sucrose (pH, 5.1) in sterile water twice to remove any unencapsulated GSH. Liposome pellets were resuspended in 300 mM ammonium sulfate (pH, 5.1) containing 300 mM sucrose in sterile water and stored at 4°C until needed.

After manufacture, the particle size of the liposomes was measured at 488-nm with a DynaPro Dynamic Light Scattering system (Wyatt Technology) and found to be 108.0 ± 26.4 nm in diameter. Phospholipid content was measured using the Stewart assay.²² Total

lipid concentration of the liposomes was 60 mM. Liposomes were also assayed for endotoxin levels and bacterial growth. No growth of bacteria was detected during 14-day culture, and endotoxin levels were <2.5 EU/mL.

Preparation of ^{99m}Tc -BMEDA and ^{99m}Tc -Liposomes

The method to prepare ^{99m}Tc -BMEDA was the same as described by Wang et al,¹⁶ with minor modification. In brief, 50 mg of sodium glucoheptonate (GH; Sigma) and 3.5 μL of BMEDA (ABX Biochemicals) were combined in the same vial, followed by the addition of 5.0 mL normal saline for injection (nitrogen gas-flushed in advance). After 20 min of magnetic stir mixing, 60 μL of freshly prepared stannous chloride (Sigma) solution (15 mg/mL) was added to the GH-BMEDA solution, followed by pH adjustment using 0.05 M sodium hydroxide to achieve a pH ranging from 7 through 8. At this time, 1.0 mL of the resulting solution was transferred to a glass vial containing 0.6 mL of 2.22 GBq (60 mCi) of ^{99m}Tc -pertechnetate (GE Healthcare). The mixed solution was incubated at 25°C for 20 min with intermittent gentle shaking. The labeling efficiency of ^{99m}Tc -BMEDA was more than 80% from paper chromatography eluted with methanol or saline. The ^{99m}Tc -BMEDA was then used immediately for liposome labeling.

For the labeling study, 2.0 mL of liposomes (60 mM total lipids) were eluted through a PD-10 column (GE Healthcare) with phosphate-buffered saline (PBS; pH, 7.4) to create an ammonium sulfate pH gradient by removing free ammonium sulfate from liposome exterior and create GSH-ammonium pH gradients. The product was added to the ^{99m}Tc -BMEDA solution and incubated at 37°C for 1 h. The ^{99m}Tc -liposomes were separated from any unencapsulated ^{99m}Tc -BMEDA by elution through a PD-10 column eluted with PBS (pH, 7.4). The labeling efficiency of the liposomes with ^{99m}Tc was 62%.

^{186}Re -Liposome Preparation

The preparation of ^{186}Re -liposomes followed the previously reported protocol with minor modification.^{11,15} In brief, 1.0 mL of saline solution (pH, 5.0) containing 1.34 mg BMEDA, 22.3 mg glucoheptonate, and 1.6 mg stannous chloride was placed in a new vial, flushed with nitrogen gas, and sealed. ^{186}Re -perrhenate solution (8.5 GBq [229 mCi]; University of Missouri Research Reactor) was added to the GH-BMEDA-stannous chloride solution and incubated at 80°C for 1 h. After incubation, the ^{186}Re -BMEDA solution was cooled to room temperature and adjusted to a pH of 7.0.

Immediately before radiolabeling, 2.0 mL of liposomes (60 mM total lipid concentration) were eluted with PBS (pH, 7.4) through a PD-10 column to remove free ammonium sulfate and create GSH-ammonium pH gradients. Eluted liposomes were added to the ^{186}Re -BMEDA solution containing 4.25 GBq (115 mCi) ^{186}Re -activity and incubated for 1 h at 37°C. The ^{186}Re -liposomes were separated from any free radionuclide by PD-10 column

chromatography eluted with PBS (pH, 7.4). The mean labeling efficiency of ^{186}Re -liposomes for 3 separate cohort studies was $77.3\% \pm 5.7\%$.

MRI

The animals underwent pretreatment MRI at 21 days after inoculation of the GBM cells. MRI was then repeated at 35, 50, and 71 days after treatment. MRIs were acquired in a small animal Bruker Biospec 7-Tesla MR scanner (Bruker) equipped with a 72 mm linear volume coil for transmit and a rat brain surface receive coil. The animals were sedated with 3% isoflurane in 100% oxygen and maintained at 1.5% during the procedure. A circulating water warming blanket regulated by a digi-sense controller was wrapped around the animal. The tail vein was catheterized with a small gauge needle, through which 0.25 mL/350 g of intravenous contrast material (gadodiamide, Omniscan; Nycomed-GE Healthcare) was injected. Postgadolinium T1-weighted images were acquired in the transverse plane with the following parameters: TE 4.058 ms; TR 300 ms; number of signals acquired, 6; section thickness, 0.43 mm; field of view, 2.56 cm $\times$ 2.56 cm; matrix, 128 $\times$ 128. Fifteen slices were acquired with an in-plane resolution of 200 μm $\times$ 200 μm . MRI studies were interpreted on Mango freeware (ric.uthscsa.edu/mango). Each study was interpreted blindly by a neuro-radiologist (J.E.G.) with more than 15 years of experience and a fourth year diagnostic radiology resident (D.V.). Tumor areas were calculated using the product of the maximum and perpendicular diameters, and care was taken to exclude the area of radiation-induced gliosis and edema that surrounded the enhancing lesion.

Pilot ^{99m}Tc -liposome Imaging Studies

To establish the correct volume of administration, ^{99m}Tc -liposomes were intracranially administered in glioma tumors of rats placed in the stereotactic frame by CED in volumes of 50 and 100 μL at 2 $\mu\text{L}/\text{min}$ and imaged by SPECT/CT (Figs 1 and 2). Planar gamma camera, SPECT, and CT images were acquired using a dual head micro-SPECT/CT scanner (XSPECT; Gamma Medica). Anterior-posterior static images were acquired at 1 h, 2 h, 4 h, and 20 h (2, 2, 2, and 10 min acquisitions, respectively) using low-energy high-resolution parallel hole collimators. The percentage of ^{99m}Tc -activity retained in the tumor, and the surrounding tissues across time were calculated from region of interest (ROI) of static images using a standard ^{99m}Tc -source positioned outside the animal but still within the field of view as a point of reference.

At 2 hours after the intratumoral injection and immediately after static image acquisition, 1-mm pinhole collimator SPECT images were acquired, with the center of the field of view focused on the tumor of each animal (dual detectors: 32 projections/detector, 30 s/projection). The radius of rotation was maintained at approximately 4.1 cm with a field of view of approximately

Fig. 1. Planar gamma camera images acquired with a high-resolution parallel hole collimator of glioblastoma-bearing rats after intratumoral administration of either a 50 μ L volume (A–D) or 100 μ L volume (E–H) of ^{99m}Tc -liposomes at 1 h (A and E), 2 h (B and F), 4 h (C and G), and 20 h (D and H) after administration. The 1 h image (A) demonstrates the site of the administration (yellow arrow) and an image standard of known ^{99m}Tc -activity (white arrow). By 1 h (A), there was some loss of ^{99m}Tc -activity into the spinal canal that increases in the rat administered with 100 μ L (E). After this initial loss of activity, good retention was observed at the tumor site at 2–20 h (B–D, F–H). Coregistered SPECT/CT 3D volume images of glioblastoma-bearing rats acquired 2 hours after intratumoral administration of 50 μ L (I) or 100 μ L (J) volume of ^{99m}Tc -liposomes. With the 50 μ L volume (I), a small amount of ^{99m}Tc -activity can be visualized to have moved anterior and posterior to the site of injection. With the 100 μ L volume (J), a larger amount of activity is visualized posterior to the site of injection.

Fig. 2. Planar gamma camera images of a glioblastoma-bearing rat after intratumoral administration of a 25 μ L volume of ^{186}Re -liposomes acquired simultaneously with either a parallel hole collimator (A–E) or 1-mm single pinhole collimator (F–J) at 0.5 h (A and F), 4 h (B and G), 24 h (C and H), 48 h (D and I), and 96 h (E and J) after administration. The 0.5 h image (A) demonstrates the site of the administration (yellow arrow) and an image standard of known ^{186}Re -activity (white arrow). The administered activity remained confined to the site of injection, with good retention over a 96-hour period in comparison with the image standard.

8.02 cm. SPECT imaging was followed by CT image acquisition (X-ray source: 75 KVp, 280 mA; 256 projections) at the same position of the animal. Software provided with micro-SPECT/CT was used for image reconstruction and SPECT/CT image fusion. SPECT images were reconstructed to produce image sizes of $80 \times 80 \times 80$ with an image resolution of 0.95 mm. CT images were reconstructed, resulting in image sizes of $512 \times 512 \times 512$ with a 0.15 mm image resolution.

Treatment Study With Intratumoral Infusion of ^{186}Re -Liposomes and Nonradioactive Liposome Formulations

The U87 GBM-bearing nude rats were divided into control and treatment groups. On day 23 after implantation of the GBM tumor cells, rats were placed in a stereotactic frame, and a needle was placed in the same location and depth at which the tumor cells were implanted. The control animals were infused with non-radioactive liposomes of the same size, lipid formulation, and lipid dose used with the ^{186}Re -liposomes, and the treated rats were infused with ^{186}Re -liposomes of varying radioactivity doses from 25 μCi to 125 μCi . The volume of liposomes infused in both control and treatment rats was 25 μL liposomes at a rate of 2 $\mu\text{L}/\text{min}$. The needle was left in place for 5 min and then gradually withdrawn. For the U251 GBM-bearing nude rats, animals were handled identically with the exception of treatment on day 14 because of the more rapid growth rate.

Imaging, Image Analysis, and Radiation Absorbed Dose Calculation for ^{186}Re -Liposomes

Retention and distribution of the ^{186}Re -liposomes was monitored using planar gamma camera imaging. Immediately after intratumoral infusion of the ^{186}Re -liposomes and at 4, 24, 48, and 96 h after infusion (5, 10, 15, 20, and 30 min acquisition times, respectively), the glioma rats were placed prone in the scanner. Static images were acquired simultaneously with both a parallel hole collimator located on 1 camera head and 1 mm-pinhole collimator focused on the brain on the other camera head. Image analysis and radiation absorbed dose calculation were performed as in previous studies.^{11,15} The retention of ^{186}Re activity within the GBM tumor area and the distribution of activity were determined using ASIPro software (Siemens Medical Solutions). The percentage of injected activity in each ROI of each planar parallel hole image was calculated from the injected activity and the activity of the ^{186}Re -standard. After decay correction, the cumulative radioactivity in the ROI is represented by the area under the curve and can be used to calculate the average radiation absorbed dose in the tumor by the following equation, assuming a negligible contribution by the penetrative gamma energy:²³

$$D(\text{Gy}) = 7.31(\text{Gy} \cdot \text{g} / (\text{mCi} \cdot \text{h})) \times \tilde{A}(\text{mCi} \cdot \text{h}) / m(\text{g})$$

In the above equation, D is the radiation absorbed dose in Gy, $\tilde{A}$ is the cumulative radioactivity as described above in $\text{mCi} \cdot \text{h}$, and m is the weight of the tumor tissue in g as calculated from the pretreatment MRI.

Results

Liposome Volume and Retention

To allow for a reasonable time for an effect of treatment to intervene on tumor-related death and improve survival, treatment was planned for day 21 after inoculation. On the basis of our experience with the U87 intracranial xenografts, this time point correlated to an approximately 3-mm lesion occupying an approximate 100 μL volume. We therefore felt that this volume empirically represented the appropriate treatment volume. To better characterize the retention of this volume of liposomes in our animal model and the ability to deliver an appropriate field of therapy, we performed intratumoral infusions of technetium-99m-labeled liposomes, followed by SPECT/CT imaging. In planar image quantitative analysis, 50%–65% of the injected activity was observed as a discrete focus at the site immediately after injection (Fig. 1). The $^{99\text{m}}\text{Tc}$ -activity was reasonably retained at the site of injection at 2, 4, and 20 h after injection with approximately 50% of the $^{99\text{m}}\text{Tc}$ -activity remaining at the site of the injection. Although retention overall was good, there unfortunately was a significant amount of $^{99\text{m}}\text{Tc}$ -activity also observed in the cerebellum and brainstem (Fig. 1J). Given potential toxicity, a lower volume of 50 μL was also evaluated and showed equivalent retention over time, while providing adequate coverage without extension into the posterior fossa (Fig. 1I). A volume of 50 μL was therefore felt to represent the upper limit, and further experiments were performed at 25 μL volume to allow for variability in tumor size. These experiments were then repeated with ^{186}Re -liposomes in a volume of 25 μL , which showed excellent localization of ^{186}Re -activity and retention through 96 hours (Fig. 2).

Maximum Tolerable Dose

Although others had previously demonstrated the safe administration of up to 12 Gy rhenium-labeled nanoparticles injected into the brain,¹² the higher limits of tolerability have not been well described. In addition, determination of the total safe dose administered can only be determined using highly specific activity labeling methods. We therefore sought to characterize the tolerability of higher doses generated using our highly efficient ^{186}Re -loading process. An initial pilot experiment was performed with doses administered from 220 to 560 Gy ($n = 6$) to compare survival with controls ($n = 6$). Of interest, although 2 animals at the lower doses died at 19 and 22 days after treatment, animals treated at the highest doses survived through sacrifice on day 37 after treatment without weight loss, neurological deficit, or other evidence of toxicity. Histologic analysis by a neuropathologist showed no evidence of residual

Fig. 3. Luciferase emission quantification as a surrogate for tumor volume (A) from animals bearing U87 xenografts and (B) bearing U251 xenografts (mean values at time point with standard deviation indicated by error bars).

tumor. We then progressively extended the dosing range in 2 successive cohorts with administration of 18–1845 Gy (12 rats in the treatment group and 8 controls), allowing animals to live 120 days after treatment before sacrifice. We consistently observed in these cohorts that the animals tolerated all doses without evidence of toxicity, and no maximum tolerable dose was reached. Only one animal died prematurely (1600 Gy), and this was associated with a diffuse rash and what was felt by the veterinary staff to represent a pre-existing infection.

Tumor Growth

Xenografts were followed by both noninvasive bioluminescence luciferase imaging and MRI to determine whether a dose-dependent effect could be observed. In fact, as demonstrated in Fig. 3A, a separation in bioluminescence between animals treated with ≤ 100 Gy and those treated with higher doses was seen within 7 days of treatment. This became statistically significant by 11 days after treatment ($\chi^2[1, N = 19] = 4.8$, $P = .029$), at which time 11 treated and all 8 control rats were alive. The separation in bioluminescence further continued thereafter. In addition, many of the rats had a loss of luciferase activity to background levels suggestive of a complete eradication of tumor (Fig. 3A). Because some animals lost all luciferase activity and the response in others was more progressive at lower doses, the deviation increased. Repeat experiments performed in an identical fashion with the exception of all animals being treated at high dose (mean of 1700 Gy) in the more invasive U251 line, as demonstrated in Fig. 3B, showed statistically significant clear separation in bioluminescence at 6 days after treatment ($\chi^2[1, N = 9] = 4.7$, $P = .029$). MRI showed a trend ($\chi^2[1, N = 6] = 2.456$; $P = .117$) toward reduced tumor size at 13 days after treatment, as shown in Fig. 4, but was not statistically significant because many of the control animals had already died and could not be included in the analysis. Example images of marked differences in sizes appreciated between the groups is illustrated in Fig. 5.

Fig. 4. Cross-sectional area of tumors calculated from MRI was initially equivalent at treatment but then appeared to separate after treatment.

Animal Survival

Control rats ($n = 14$) uniformly had a median survival time of 49 days in each of the 3 experiments (95% confidence interval [CI], 44–53 days) from intracranial injection until clinical signs of neurologic compromise (dull, listless, moribund) or death. In intent-to-treat analysis without regard to dose, animals treated with ^{186}Re -liposomes had a median survival of 126 days (95% CI, 78.4–173 days), mainly resulting from sacrifice after meeting prescribed endpoints as needed for histologic analysis. Log-rank analysis between these 2 groups was highly significant ($P = .0013$). When survival length by dose was analyzed, there was a recognizable separation beginning at a dose of 100 Gy. Survival analysis for animals treated with more than 100 Gy, compared with less or none (Fig. 6A), with log-rank analysis had even greater significance ($P < .0001$). In a similar experiment with U251 xenografts treated with a mean dose of 1700 Gy, results were even more impressive, with a median survival would have surpassed 120 days because of lack of mortality (Fig. 6B), and log-rank analysis of survival was highly significant ($P = .0057$).

Fig. 5. Contrast-enhanced MRI of intracranial tumors reveals comparable pretreatment tumor size with progressive growth by day 14 in controls, whereas ^{186}Re treated tumors progressively disappear over time.

Histology

Brains were collected from sacrificed animals and subjected to standard histological staining and blinded review by a neuropathologist. Because some animals died spontaneously before developing obvious neurologic signs, not all brains could be collected in a timely fashion and were therefore not included in the analysis. Immunohistochemistry for GFAP, vimentin, synaptophysin, Ki-67, and CD68 was performed on samples at the discretion of the pathologist per standard clinical laboratory technique. Because U87 cells are poorly differentiated and negative for both GFAP and synaptophysin, this allowed differentiation of tumor cells from surrounding gliosis (Fig. 7). Of those brains that could be analyzed, blinded analysis revealed that all treated animals ($n = 7$) had no residual tumor (Table 1). Some minimal areas of necrosis were identified in a few specimens, with a mild rim of surrounding gliosis and scattered CD68 + macrophages (Fig. 7). Macrophages were also noted at the invading edge of tumors. These findings first suggest that doses of radiation at 100 Gy or more can lead to tumor eradication in this model. Second, these results suggest that ^{186}Re -liposome convection-enhanced brachytherapy can selectively deliver higher doses of radiation to the tumor without the overt toxicity typically seen with external beam radiotherapy or X-ray brachytherapy used at similar doses.²⁴ Of the animals that were untreated and included ($n = 3$), large areas of tumor were identified with abundant mitotic figures and Ki-67 staining of approximately 50%.

Discussion

Radiation therapy remains an essential component of treatment for most malignancies, including primary

Fig. 6. Animal survival (A) from rats bearing U87 xenografts as a function of treatment of less than or greater than 100 Gy and (B) from animals bearing U251 xenografts.

brain tumors. Theoretically, any tumor can be controlled if a sufficient dose of radiation is delivered to the tumor. The main limiting factor in delivering a tumoricidal dose is the toxicity to surrounding normal tissue. With standard techniques used today, even accounting for more conformal applications, doses are limited to less than 80 Gy.²⁵ Two main radiobiological

Fig. 7. Microscopic images of a brain from a treated rat reveals (A) sterilized tumor radius with incomplete necrosis and nonspecific cellular reaction to injury without evidence of residual tumor, H&E, 100 $\times$; (B) Higher power, H&E, 400 $\times$; (C) Sterilized tumor radius with gliosis, GFAP, 400 $\times$; (D) Sterilized tumor radius with occasional macrophages, CD68, 400 $\times$; (E) Tumor from an untreated animal shows macrophages at the infiltrative edge, CD68, 400 $\times$.

Table 1. Comments received by the reviewing neuropathologist (J.H.) who was blinded to treatment

Dose	Neuropathologist's Blinded Findings
0	Marked residual tumor with frequent mitotic figures and increased Ki-67 (50%)
0	Marked residual tumor with frequent mitotic figures and increased Ki-67 (50%)
0	Large well-marginated tumor with frequent mitotic figures and brisk Ki-67 staining (50%)
425	Focus of necrosis with a surrounding rim of gliosis, and scant CD68 positivity
570	No residual tumor appreciated. Minimal (if any) necrosis.
725	Negative for tumor but mild gliosis. Scant CD68 positivity.
1200	Gliosis with rarefaction of tissue, capillary neovascularity, siderophages, no residual tumor
1420	No residual tumor appreciated, mild necrosis with residual gliosis at the edge
1591	Small focus of necrosis, rim of infrequent CD68 positive cells at the edge
1845	No residual tumor appreciated. Minimal (if any) necrosis. Scattered CD68 positivity

Performance of immunohistochemistry was at the discretion of the pathologist.

differences can be noted between the approach used in this experimental model and conventional radiotherapy. First, because traditional X-ray radiation is absorbed by the body and shows an exponential decrease in the dose delivered with tissue depth, our model's placement of the radiation source into the tumor and retention at that site mediated by liposomes results in the bulk of radiation being absorbed by the tumoral tissue rather than by the surrounding normal brain tissue. Second, although ^{186}Re releases some gamma rays which allow for external imaging, the bulk of the emission is in the form of beta particles that have a short path length. In fact, nearly 99% of the dose is absorbed within the first 2 mm. This suggests that focal ablation could be achieved at the injection site with little risk to

the surrounding brain. In fact, ^{186}Re -liposome convection-enhanced brachytherapy was achieved with markedly increased doses up to 1845 Gy without overt evidence of toxicity. This supports the conclusion that the ^{186}Re -liposome radioactivity was restricted primarily to tumor tissue. This is also significantly different from the toxicity previously reported with ^{188}Re , which has more than twice the path length, and showed toxicity at doses as low as 12 Gy.¹²

The significant efficacy observed in this study, with irradiation of tumors on histology despite the short path length of the ^{186}Re , raises an important potential difference between liposomal brachytherapy and more static forms of brachytherapy, such as the X-ray emitting palladium seeds and gliastite. Recent studies in

which liposomal nanoparticles were intratumorally administered into glioma tumors have shown that 60%–80% of the nanoparticles became localized in the abundant microglia and macrophages, and these mobile phagocytic cells were shown to have significant potential to carry and move encapsulated drugs within brain tumors.²⁶ Intratumoral phagocytic cells, such as the macrophages that we observed along the invasive margin of tumors, could significantly improve the distribution of beta-particle emitting therapeutic radionuclides in the tumor and provide more specific therapy to infiltrative tumor cells in ways not possible with other fixed forms of brachytherapy. Thus, although the ¹⁸⁶Re has a short path length of only a few millimeters, it likely circulates in the tumor through microglia to provide more conformal coverage while limiting exposure of the normal adjacent tissue.

An additional attribute of this potential therapy, evident in noninvasive SPECT imaging, is the capacity for real-time image feedback and iterative modulation of nanoparticle brachytherapy with real-time dosimetry calculations of the delivered ¹⁸⁶Re-liposomes to provide the most accurate treatment and ensure complete tumor coverage. In addition, similarly to current I-131 therapy for thyroid cancer, retreatment would be possible because of the fact that very low doses are delivered to the surrounding normal brain tissue. Human studies of brachytherapy with highly specific activity rhenium nanoparticles, such as those used in this experiment,

are needed to better characterize their safety, efficacy, and application.

Acknowledgments

We thank Cristina Santoyo, Ricardo Perez III, and Dr. Shihong Li for technical assistance during the SPECT/CT imaging studies and Yongjian Ouyang and Dr. Joel Michalek with UTHSCSA Department of Epidemiology and Biostatistics. Liposome particle size was determined in the UTHSCSA Center for Macromolecular Interactions, which is supported in part by the Cancer Therapy and Research Center through the National Institutes of Health and by Texas state funds provided through the Office of the Vice President for Research.

Conflict of interest statement. None declared.

Funding

The work was supported by the UTHSCSA Cancer Therapy and Research Center through the National Institutes of Health, a National Cancer Institute P30 award (CA054174), and by Texas State funds provided through the Office of the Vice President for Research of the UTHSCSA.

References

1. CBTRUS: Central Brain Tumor Registry of the United States. 2005–2006 Primary Brain Tumors in the United States Statistical Report. 1998–2002 Years Data Collected. <http://www.cbtrus.org/reports//2005-2006/2006report.pdf>.
2. Hou LC, Veeravagu A, Hsu AR, Tse VC. Recurrent glioblastoma multiforme: a review of natural history and management options. *Neurosurg Focus*. 2006;20(4):E5.
3. Stupp R, Mason WP, van den Bent MJ, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med*. 2005;352(10):987–996.
4. Penny KS, Philip HG, David AL, et al. Patterns of recurrence of glioblastoma multiforme after external irradiation followed by implant boost. *International Journal of Radiation Oncology, Biology, Physics*. 1994;29(4):719–727.
5. Bobo RH, Laske DW, Akbasak A, Morrison PF, Dedrick RL, Oldfield EH. Convection-enhanced delivery of macromolecules in the brain. *Proc Natl Acad Sci USA*. 1994;91(6):2076–2080.
6. Debinski W, Tatter SB. Convection-enhanced delivery for the treatment of brain tumors. *Expert Rev Neurother*. 2009;9(10):1519–1527.
7. Kikuchi T, Saito R, Sugiyama S, et al. Convection-enhanced delivery of polyethylene glycol-coated liposomal doxorubicin: characterization and efficacy in rat intracranial glioma models. *J Neurosurg*. 2008;109(5):867–873.
8. Krauze MT, Vandenberg SR, Yamashita Y, et al. Safety of real-time convection-enhanced delivery of liposomes to primate brain: a long-term retrospective. *Exp Neurol*. 2008;210(2):638–644.
9. Vinchon-Petit S, Jarnet D, Paillard A, Benoit JP, Garcion E, Menei P. In vivo evaluation of intracellular drug-nanocarriers infused into intracranial tumours by convection-enhanced delivery: distribution and radiosensitisation efficacy. *J Neurooncol*. 2010;97(2):195–205.
10. Krauze MT, Forsayeth J, Yin D, Bankiewicz KS. Convection-enhanced delivery of liposomes to primate brain. *Methods Enzymol*. 2009;465:349–362.
11. French JT, Goins B, Saenz M, et al. Interventional therapy of head and neck cancer with lipid nanoparticle-carried rhenium 186 radionuclide. *J Vasc Interv Radiol*. 2010;21(8):1271–1279.
12. Allard E, Hindre F, Passirani C, et al. 188Re-loaded lipid nanocapsules as a promising radiopharmaceutical carrier for internal radiotherapy of malignant gliomas. *European Journal of Nuclear Medicine and Molecular Imaging*. 2008;35(10):1838–1846.
13. Bao A, Goins B, Klipper R, Negrete G, Mahindaratne M, Phillips WT. A novel liposome radiolabeling method using 99mTc-“SNS/S” complexes: in vitro and in vivo evaluation. *J Pharm Sci*. 2003;92(9):1893–1904.
14. Bao A, Goins B, Klipper R, Negrete G, Phillips WT. 186Re-liposome labeling using 186Re-SNS/S complexes: in vitro stability, imaging, and biodistribution in rats. *J Nucl Med*. 2003;44(12):1992–1999.
15. Wang SX, Bao A, Herrera SJ, et al. Intraoperative 186Re-liposome radionuclide therapy in a head and neck squamous cell carcinoma xenograft positive surgical margin model. *Clin Cancer Res*. 2008;14(12):3975–3983.

16. Wang SX, Bao A, Phillips WT, et al. Intraoperative therapy with liposomal drug delivery: retention and distribution in human head and neck squamous cell carcinoma xenograft model. *Int J Pharm*. 2009;373(1–2):156–164.
17. Zavaleta CL, Goins BA, Bao A, McManus LM, McMahan CA, Phillips WT. Imaging of ¹⁸⁶Re-liposome therapy in ovarian cancer xenograft model of peritoneal carcinomatosis. *J Drug Target*. 2008;16(7):626–637.
18. Chang YJ, Chang CH, Chang TJ, et al. Biodistribution, pharmacokinetics and microSPECT/CT imaging of ¹⁸⁸Re-bMEDA-liposome in a C26 murine colon carcinoma solid tumor animal model. *Anticancer Res*. 2007;27(4B):2217–2225.
19. Chen LC, Chang CH, Yu CY, et al. Biodistribution, pharmacokinetics and imaging of (¹⁸⁸Re)-BMEDA-labeled pegylated liposomes after intraperitoneal injection in a C26 colon carcinoma ascites mouse model. *Nucl Med Biol*. 2007;34(4):415–423.
20. Chang YJ, Chang CH, Yu CY, et al. Therapeutic efficacy and microSPECT/CT imaging of ¹⁸⁸Re-DXR-liposome in a C26 murine colon carcinoma solid tumor model. *Nucl Med Biol*. 2010;37(1):95–104.
21. Lal S, Lacroix M, Tofilon P, Fuller GN, Sawaya R, Lang FF. An implantable guide-screw system for brain tumor studies in small animals. *J Neurosurg*. 2000;92(2):326–333.
22. Stewart JCM. Colorimetric Determination of Phospholipids with Ammonium Ferrothiocyanate. *Anal Biochem*. 1980;104(1):10–14.
23. Eckerman KF, Endo A, Society of Nuclear Medicine (1953-), Society of Nuclear Medicine (1953-). Medical Internal Radiation Dose Committee. MIRDO: radionuclide data and decay schemes. 2nd ed. Reston, VA: Society of Nuclear Medicine; 2007.
24. Kondziolka D, Lunsford LD, Claassen D, Maitz AH, Flickinger JC. Radiobiology of radiosurgery: Part I. The normal rat brain model. *Neurosurgery*. 1992;31(2):271–279.
25. Werner-Wasik M, Scott CB, Nelson DF, et al. Final report of a phase I/II trial of hyperfractionated and accelerated hyperfractionated radiation therapy with carmustine for adults with supratentorial malignant gliomas: Radiation Therapy Oncology Group Study 83-02. *Cancer*. 1996;77(8):1535–1543.
26. Alizadeh D, Zhang L, Hwang J, Schluep T, Badie B. Tumor-associated macrophages are predominant carriers of cyclodextrin-based nanoparticles into gliomas. *Nanomedicine*. 2010;6(2):382–390.