ABSTRACT

Success of boron neutron capture therapy (BNCT) is dependent on cellular and molecular targeting of sufficient amounts of boron-10 to sustain a lethal 10B(n, a)7Li capture reaction. The purpose of the present study was to determine the efficacy of boronated epidermal growth factor (EGF) either alone or in combination with boronophenylalanine (BPA) as delivery agents for an epidermal growth factor receptor (EGFR)-positive glioma, designated F98EGFR. A heavily boronated precision macromolecule [boronated starburst dendrimer (BSD)] was chemically linked to EGF by heterobifunctional reagents. Either F98 wild-type (F98WT) receptor (–) or EGF gene-transfected F98EGFR cells, which expressed 5 × 10⁴ receptors/cell, were stereotactically implanted into the brains of Fischer rats, and 2 weeks later biodistribution studies were initiated. For biodistribution studies rats received an intratumoral (i.t.) injection of 125I-labeled BSD-EGF and were euthanized either 6 or 24 h later. At 6 h, equivalent amounts of BSD-EGF were detected in F98EGFR and F98WT tumors. Persistence of the bioconjugate in F98EGFR tumors was specifically determined by EGFR expression. By 24 h 33.2% of injected dose/g of EGF-BSD was retained by F98EGFR gliomas compared with 9.4% % of injected dose/g in F98WT gliomas, and the corresponding boron concentrations were 21.1 μg/g and 9.2 μg/g, respectively. Boron concentrations in normal brain, blood, liver, kidneys, and spleen were at nondetectable levels (<0.5 μg/g). On the basis of these results, BNCT was initiated at the Brookhaven National Laboratory Medical Research Reactor. Two weeks after implantation of 10⁶ F98EGFR or F98WT tumor cells, rats received an i.t. injection of BSD-EGF (~60 μg 10B/15 μg EGF) either alone or in combination with i.v. BPA (500 mg/kg). Rats were irradiated at the Brookhaven Medical Research Reactor 24 h after i.t. injection, which was timed to coincide with 2.5 h after i.v. injection of BPA for those animals that received both capture agents. Untreated control rats had a mean survival time (MST) ± SE of 27 ± 1 day, and irradiated controls had a MST of 31 ± 1 day. Animals bearing F98EGFR gliomas, which had received i.t. BSD-EGF and BNCT, had a MST of 45 ± 5 days compared with 33 ± 2 days for animals bearing F98WT tumors (P = 0.0032), and rats that received i.t. BSD-EGF in combination with i.v. BPA had a MST of 57 ± 8 days compared with 39 ± 2 days for i.v. BPA alone (P = 0.016). Our data are the first to show in vivo efficacy of BNCT using a high molecular weight boronated bioconjugate to target amplified EGFR expressed on gliomas, and they provide a platform for the future development of combinations of high and low molecular weight agents for BNCT.
glioma, which had been transfected with the human gene encoding EGFR (22). However, for therapy studies of experimental brain tumors, it is preferable to have a syngeneic tumor model (23). Therefore, in the present study we have developed a tumor model in which the parental or wild-type F98 glioma, designated F98_WT, which is syngeneic to Fischer CD rats and EGFR(−), was transfected with the human gene encoding EGFR. After in vitro and in vivo characterization of this tumor, designated F98_EGFR, we have evaluated the efficacy of BNCT after i.t. injection of BSD-EGF with or without i.v. administration of BPA to rats bearing either F98_EGFR or F98_WT gliomas. As described in detail in the following report, i.t. injection of BSD-EGF, either alone or in combination with i.v. administration of BPA, followed by BNCT, resulted in a significant prolongation in survival time of F98_EGFR glioma-bearing rats.

MATERIALS AND METHODS

Transfection of F98 Glioma Cells with Human EGFR Gene. Human EGFR cDNA was cloned by reverse transcription-PCR from A431 cells (24). PCR primers with engineered restriction sites corresponding to the full-length EGFR coding sequence were used. PCR was performed with 1.5 units Taq (Life Technologies, Inc., Grand Island, NY) and 0.075 μM plaque-forming units of proofreading polymerases (Stratagene, La Jolla, CA) for 30 cycles, annealing at 60°C, and extension at 72°C. After isolating the PCR fragment by Topo-cloning (Invitrogen, Hercules, CA) into a TA vector for sequencing, the cDNA was excised by treatment with EcoRV and XhoI restriction enzymes (25). The excised fragment was inserted into the polylinker of pLXIN (Clontech Laboratories, Inc., Palo Alto, CA) at Hpal-Xhol digested sites. pLXIN is a bicistronic vector expressing message of the cDNA insert from a cytomegalo-virus promoter linked by an iron response element sequence to the Neo-resistance gene. This provided one mRNA super transcript containing both genes, but it is translated independently (i.e., not a fusion protein), which allowed for a more robust selection of clones. pLXIN-EGFR was then transfected into F98 cells by LipofectAMINE (Life Technologies, Inc., and) and selected in G418 (Invitrogen) at a concentration of 600 μg/ml for 14 days. Colonies were selected individually using cloning discs (Bell-Art Products, Pequannock, NJ). G418-resistant colonies were screened by Western blot analysis for expression of full-length EGFR protein as follows. Cells were lysed in radioimmunoprecipitation buffer containing 50 mM TRIS-HCl (pH 7.5), 0.25% sodium deoxycholate, 1% NP40, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM EGTA, and 1 μg/ml each of pepstatin, leupeptin, and aprotinin. Lysates were sonicated for 30 s and centrifuged to remove debris. Extracts (100 μg) were boiled for 5 min and applied to 7.5% SDS-PAGE. Proteins were electrophoretically on to Nytran membranes (Schleicher and Schuell, Keene, NH) and then probed with anti-EGFR antibody 1005 (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Protein extracts of the human epidermoid carcinoma cell line A431, which is known to express EGFR (24), was used as a positive control. Specific binding was detected by alkaline phosphatase-conjugated secondary antibodies and disodium 2-chloro-4(2-methoxy-5(1,2-dioxetan-32-5-chloro)tricyclo[3.3.1.13,7]decan-4-yl)-1-phenyl phosphate chemiluminescent substrate (Bio-Rad, Carlsbad, CA). After this, additional screening was carried out by RLF and fluorescence-activated cell sorting. The sorting of clones derived at this time were named F98iB1707 (where the number indicates the individual clone), and in this report it has been designated F98-EGFR.

EGFR binding assay. F98_WT cells were propagated in DMEM containing glucose, 1-glutamine, and 10% fetal bovine serum (Life Technologies, Inc.). F98_EGFR cells were grown in the same medium supplemented with 200 mg/ml of G418. The receptor-binding activity of EGFR with either F98_WT or F98_EGFR cells was quantified by a direct binding assay, briefly described as follows. F98 cells (5 x 10^5 cells/well) were seeded into 24-well flat-bottomed plates (Corning Inc., Corning, NY) and allowed to attach overnight, after which the cells were washed twice with serum-free DMEM and then incubated for 2 h in the same medium. DMEM then was removed and various concentrations (0–500 ng/ml) of 125I-labeled BSD-EGF were added to triplicate cultures and incubated at 20–22°C in an atmosphere containing 95% air and 5% CO2 for 2 h. The cells then were washed three times with PBS and harvested using 0.5 mM EDTA in PBS (pH 7.2), and cell-associated radioactivity was determined by γ scintillation counting using a well counter (model 1185; Trin Analytic, Elk Grove Village, IL).

Preparation and Purification of BSD-EGF Bioconjugate. A fourth-generation starburst dendrimer, which is composed of repetitive polyiodo amino groups arranged in a starburst pattern, was boronated with a boron-10 enriched (>98%) 60B methylisocyanato polyhedral borane anion, [Na(C6H3(CH3)9)2BpH2H4CO], to yield BSD using a procedure described by us in detail elsewhere (9). Briefly, the BSD was reacted with N-succinimidyl 3-(2-pyridyldithio) propionate, and the resulting product was cleaved with DTT to yield a sulfhydryl-containing BSD. Human EGFR was derivatized with the heterofunctional reagent maleimidebenzoyl-N-hydroxysulfosuccinimide ester (Pierce Chemical Co., Rockford, IL) and linked to sulfhydryl-containing BSD-EGF bioconjugate (9). The bioconjugate was purified by column chromatography using a Superdex-G-75 column and eluted with 0.1 M Tris and 0.2 M NaCl buffer (pH 8.5). Fractions (1 ml) were collected, and protein concentrations were determined spectrophotometrically by measuring absorbance at 280 nm with a Beckman DU-6 spectrophotometer (Beckman Instruments, Inc., Irvine CA). Boron was quantified by DCP-AES using a Spectraspan VB spectrometer (Applied Research Laboratories, La Brea, CA), as described previously (26). Fractions containing peak concentrations of both protein and boron were pooled and used in the studies described in the following section.

Evaluation of Tumorigenicity of F98_EGFR Glioma. To define the tumorigenicity of the F98_EGFR glioma and to compare it to the wild-type tumor, CD-Fischer rats (Charles River Laboratories, Wilmington, MA) were stereotactically implanted with F98_EGFR or F98_WT cells into the right caudate nucleus using a procedure described previously (27). Briefly, rats were sedated by i.p. administration of a 1:2:1 mixture of ketamine/xylazine at a dose of 120 mg of ketamine/20 mg xylazine/kg b.w., after which a plastic screw (Arrow Machine Manufacturing, Inc., Richmond, CA) was embedded into the skull. F98 cells were injected over 10–15 s through a central hole in the plastic screw into the right caudate nucleus at a concentration of 10^5, 10^4, or 10^5 cells/10 μl of serum-free DMEM containing 1:2:1% agarose with a gelling temperature of <30°C. The screw hole was filled with bone wax immediately after withdrawal of the needle, and the operative field was flushed with betadine before the scalp incision was closed with a single sterilized clip. The rats were observed daily and weighed three times per week after tumor implantation to monitor their clinical status. As determined in previous studies with the F98 glioma (5, 6), the combination of sustained weight loss, ataxia, and periorbital bleeding indicated that death was imminent. Therefore, to minimize discomfort animals displaying these signs were euthanized, and survival times were determined from the day of tumor implantation to euthanasia plus 1 day.

Biodistribution of 125I-BSD-EGF. BSD-EGF was reacted with Bolton-Hunter reagent to introduce a phenolic function into the bioconjugate (28). It then was radioiodinated with 125INa by a procedure described by us in detail (9) using 2 mg/ml of chloramine-T (ICN Biomedicals Inc., Costa Mesa, CA) in 0.5 M phosphate buffer (pH 7.5). 125I-labeled BSD-EGF was shown to be stable and was not dehalogenated for at least 1 week when kept at 4°C. F98_WT or F98_EGFR cells (100,000) were stereotactically implanted into the right caudate nucleus of Fischer rats, and 12 to 14 days later, biodistribution studies were initiated. Intratumoral injection of 125I-labeled BSD-EGF was performed through the central hole in the plastic screw, which was embedded at the time of tumor implantation. Rats received an i.t. injection of 5 μCi of 125I-labeled BSD-EGF (40 μg of boron/10 μg EGF) and were euthanized either 6 or 24 h later. The test agent (10 μl) was injected over 2 min with a 25-μl Hamilton syringe fitted with a 27-gauge needle. Tumor, normal brain, blood, and other tissue samples were taken, and biodistribution studies were initiated. Intratumoral injection of 125I-labeled BSD-EGF was performed through the central hole in the plastic screw, which was embedded at the time of tumor implantation. Rats received an i.t. injection of 5 μCi of 125I-labeled BSD-EGF (40 μg of boron/10 μg EGF) and were euthanized either 6 or 24 h later. The test agent (10 μl) was injected over 2 min with a 25-μl Hamilton syringe fitted with a 27-gauge needle. Tumor, normal brain, blood, and other tissue samples were taken, and biodistribution was determined by γ-scintillation counting using a well counter. Tissue samples were counted along with triplicate samples of the injectate to correct for the decay of the isotope before γ counting. In a separate study to quantify the uptake of boron in tumor and normal tissues at the time the animals were to be irradiated, nonradioiodinated BSD-EGF was injected i.t. either alone or in combination with i.v. BPA, and the animals were euthanized 24 h after administration. Boron concentrations were determined by DCP-AES (26), and the %ID/g was calculated.
administration of BPA at a dose of 800 mg/kg b.w., administered 2.5 h before irradiation. Several days after BNCT, the animals were returned to Columbus, Ohio, where their clinical status was carefully monitored, and they were weighed at daily intervals. On the basis of the observation that the animals tolerated this treatment, a definitive study was initiated. BNCT was carried out 14 days after stereotactic implantation of 103 F98 EGFR or F98 WT glioma cells. Rats were randomized into six experimental groups of 8–10 animals each. All of the animals had F98 EGFR tumors except for those in group 2, which had F98 WT tumors. Groups 1, 2, and 3 received an i.t. injection of BSD-EGF (60 μg 10 B/15 μg EGF); in addition, group 3 received an i.v. injection of BPA (500 mg/kg b.w., equivalent to 27 mg B/kg); group 4 received i.v. BPA alone; group 5 served as irradiated controls, and group 6 were untreated controls. Rats were irradiated at the BMRR 24 h after i.t. injection of BSD-EGF so as to enhance specific uptake and non-specific retention, and this was timed to coincide with 2.5 h after i.v. injection of BPA for those animals that received both capture agents.

All of the irradiated rats were anesthetized with a 1:2/1 mixture of ketamine/xylazine and placed supine in a body shield-head stabilizer, as described elsewhere (5, 6). The tumor implantation site was centered in the 1.15-cm diameter aperture of the neutron beam collimator, and animals were irradiated for 4 min at a reactor power of 2 MW to give a dose of 8 MW-min. A blood sample for boron determination was taken from each animal before irradiation. On completion of BNCT, the animals were returned to Columbus, Ohio, for observation until their time of death or euthanization. All of the animals were weighed at least three times per week after irradiation to monitor their clinical status.

To confirm that all of the animals had progressively growing tumors at the time of euthanization (or death), the brains were removed, fixed in formalin, and then cut coronally at the level of the optic chiasm, and 2 mm anterior and posterior to it. Coronal sections through the tumor were embedded in paraffin, sectioned at 4 μm, stained with H&E, and then examined microscopically to assess histopathologic changes. The tumor size index was determined by microscopic examination of H&E-stained coronal sections of the brain that subjectively had the largest cross-sectional areas of tumor. On the basis of the estimated tumor size, a semiquantitative grading scale ranging from 0 to 4 was used to score each section as follows: 0, no tumor; 1, very small, i.e., microscopic (<1 mm); 2, small (~1–3 mm); 3, large (~4–7 mm); and 4, massive (>8 mm).

**Dosimetry.** The neutron fluence was determined by multiplying the reactor power level in MW by the duration of irradiation in min to yield the dose in MW-min. Reactor power was maintained at 2 MW for 4 min (8 MW-min) during the irradiation of all of the animals. The mixed radiation field delivered to tissue during BNCT is comprised mainly of thermal neutrons, fast neutrons (>10 keV), γ photons, and heavy-charged particles (He, 1 Li, 4 He, and 14 C) from the 10 B(n, α) Li and 14 N(n, p) 14 C reactions. To determine the thermal neutron fluence, dosimetric measurements were carried out both on dead rats and a plastic phantom, with bare or cadmium-coated gold wires either attached to the skin or inserted into the plastic phantom. The fast (>10 keV) neutron and γ doses delivered to the rats were determined using paired tissue-equivalent plastic chambers (Shonka A-150 plastic; Far West Technology, Goleta, CA) with tissue-equivalent gas (Rossi gas) and graphite chambers filled with CO2. Radiation geometry, body shielding, and dosimetric parameters for rat brain tumor irradiation at the BMRR have been described in detail elsewhere (29–31). At 2-MW reactor power, the thermal neutron flux was 1.28 1010 n0,cm−2.s−1 at the head surface and 8.5 × 109 n0,cm−2.s−1 at the center of the tumor 4 mm beneath the skull. The physical dose rate (Gy/MW-min) of the radiobiologically significant beam components were: 0.039 Gy/MW-min (per μg 10 B/g) for the 10 B(n, α) Li reaction; 0.27 Gy/MW-min for the fast neutron interaction with hydrogen (1 H(n,n′p)); 0.093 Gy/MW-min for the 14 N(n,p) 14 C capture reaction that occurred with nitrogen; and 0.19 Gy/MW-min for the total γ component [beam and the 1 H(n,γ)y 1 H reaction].

**Statistical Evaluation of Data.** The means and SDs were computed for boron concentrations in the tumor, brain around tumor, ipsilateral (tumor-bearing), and contralateral (nontumor-bearing) cerebral hemispheres, and blood and the tumor:brain concentration ratios were calculated for each group. The Wilcoxon-Gehan rank-sum test (32) was used to evaluate survival data after implantation of logarithmically incremental numbers of F98 WT or F98 EGFR glioma cells. To study the effects of BNCT on survival of F98 glioma-bearing rats, the MST, SE, and median survival time were calculated for each group using the Kaplan-Meier estimate (32). Kaplan-Meier and Cox survival curves were also plotted for each group. An overall log rank test was performed to test for equality of survival curves over the six groups. The a priori hypotheses involved a comparison of i.v. BPA + i.d. BSD-EGF versus i.v. BPA and i.d. BSD-EGF, versus i.d. BSD-EGF, both in F98 EGFR glioma-bearing rats; and i.d. BSD-EGF in F98 EGFR versus F98 WT glioma-bearing rats. The Wald test was used for these comparisons, with a Bonferroni method of adjustment for the multiple comparisons (33, 34). The percentage of increased life span was determined from the following equation where ‘t’ designates treated and ‘u’ designates untreated animals:

\[
\% \text{ increased lifespan} = \frac{MeST(\text{or MST}_u) - MeST(\text{or MST}_t)}{MeST(\text{or MST}_u)} \times 100
\]

**RESULTS**

**Characterization of the F98 EGFR Rat Glioma Model.** EGFR expression was determined by Western blot analysis using anti-EGFR MoAb #1005 capable of detecting both the rat and human receptors. EGFR protein could not be detected in lysates of the F98 WT glioma, while in contrast F98 EGFR-transfected cells and human A431 cells strongly expressed human EGFR (Fig. 1). EGFR expression remained stable for 20 in vivo passages, as determined by fluorescence-activated cell sorting. Using a radioligand binding assay and Scatchard analysis, the F98 EGFR glioma expressed 5 × 10⁹ EGFR/cell compared with an undetectable number for F98 WT cells. Tumorigenicity of F98 EGFR cells was compared with that of the F98 WT glioma by implanting logarithmically incremental numbers of cells intracerebrally into syngeneic Fischer rats. All of the rats died after implantation of F98 EGFR and F98 WT glioma cells. The MST ± SD of rats after implantation of 10³, 10⁴, and 10⁵ cells were 26 ± 1, 18 ± 2, and 14 ± 2 days, respectively, for F98 EGFR cells compared with 24 ± 2, 19 ± 3, and 15 ± 2 days for the corresponding numbers of F98 WT cells (Fig. 2). There were no statistically significant differences, as determined by the Wilcoxon-Gehan rank sum test (P = 0.1), in MSTs between F98 EGFR and F98 WT glioma-bearing rats implanted with equal numbers of tumor cells.

**Biodistribution Studies.** The biodistribution of 125I-BSD-EGF after i.t. injection into either F98 EGFR or F98 WT glioma-bearing rats 12–14 days after implantation are shown in Table 1 and Fig. 3. Between 1 and 6 h after i.t. injection, 65–69% ID/g of BSD-EGF was nonspecifically localized in F98 EGFR and F98 WT tumors. However, by 24 h, 33.2% ID/g of EGF-BSD was retained by F98 EGFR gliomas compared with 9.4% ID/g in F98 WT gliomas indicating that persistence of the BSD-EGF bioconjugate in F98 EGFR tumors was determined specifically by EGFR expression. As determined by DCP-AES, at 24 h after i.t. injection BSA-EGF (60 μg of 10 B/15 μg EGF), boron concentrations were 21.1

**Fig. 1.** Western blot analysis for EGFR protein expression. Cell lysates of glioma cell lines A431 (10 μg), F98 WT (100 μg), and F98 EGFR (100 μg) were electrophoresed through 7.5% SDS-PAGE under reducing conditions and then transferred to nylon membranes. A MoAb directed against EGFR detected a 69,000 protein band corresponding to the expected molecular weight of EGFR. Glioma cell line A431, which is known to express EGFR, was used as a positive control.
µg/g or 35% ID/g of B in F98EGFR compared with 9.2 µg/g (15.3% ID/g of B) in F98WT gliomas (Table 2). The corresponding boron concentrations in the ipsilateral (tumor-bearing) cerebral hemisphere were 5.6 and 4.2 µg/g. Boron concentrations of BSD-EGF in the contralateral (nontumor-bearing) cerebral hemisphere, blood, liver, kidneys, and spleen were all at nondetectable levels (<0.5 µg/g), after i.t. injection (data not shown). After i.v. administration of BPA alone to F98EGFR glioma-bearing rats (24 h), the tumor boron concentration was 20.8 µg/g, and when administered in combination with i.t. BSD-EGF it was 43.6 µg/g (Table 2). The corresponding normal brain concentrations were 4.6 and 8.9 µg/g, respectively, and blood concentrations were 6.2–6.4 µg/g.

**BNCT**. All of the animals in the pilot study to determine tolerance to BNCT after i.t. injection of BSD-EGF lost weight within 7–10 days after treatment. Rats that received 80 µg of BSD-EGF i.t. and 800 mg/kg b.w. of BPA i.v. lost 18% of their b.w. and never regained their pretreatment weight. Animals that received either 40 or 80 µg of BSD-EGF alone or 40 µg of BSD-EGF in combination with 800 mg/kg of BPA i.v. lost <10% of their b.w. (5.6, 9.2, and 9.8%, respectively) but regained their pretreatment weights within 2 weeks. On the basis of these results, an intermediate concentration of i.t. BSD-EGF (60 µg 10B/15 µg EGF) alone or in combination with i.v. BPA (500 mg/kg b.w.) was used. BNCT was initiated at the BMRR 14 days after intracerebral implantation of 10⁶ F98EGFR glioma cells. All of the rats tolerated BNCT without any untoward effects, and 2–5 days later they were returned to Columbus, Ohio. Survival data after BNCT are summarized in Table 3, and Kaplan-Meier and Cox survival plots for BNCT-treated animals and the irradiated controls are shown in Fig. 4 and 5. Untreated and irradiated control rats had MST ± SE of 27 ± 1 day and 31 ± 1 day, respectively. The modest increase in survival of the latter group was attributable to the fast neutron, γ photon, and nitrogen capture doses that were given during irradiation. Animals bearing F98EGFR gliomas that received i.t. BSD-EGF and BNCT had a MST of 45 ± 5 days (range 32–87 days) compared with 33 ± 2 days (range 27–42 days) in animals bearing F98WT tumors. Animals that received i.t. BSD-EGF in combination with i.v. BPA had a MST of 57 ± 8 days (range 35–114 days) compared with 39 ± 2 days (range 31–46 days) for i.v. BPA alone (P < 0.01). The corresponding percentage of increase in life span were 111% for the combination versus 67% for i.t. BSD-EGF and 44% for i.v. BPA. The test for equality of the survival curves indicated that overall, the differences were highly significant (P < 0.0001). The results from the comparisons indicate that i.t. BSD-EGF + i.v. BPA was significantly different from the i.v. BPA group (P = 0.016) but not from the i.t. BSD-EGF group (P = 0.15). This lack of statistical significance was attributable to the wide range in survival times for animals that received i.t. BSD-EGF alone (32–87 days) versus those that received i.t. in combination with i.v. BPA (35–114 days). The difference in survival time for F98EGFR versus F98WT glioma-bearing rats, which had received BSD-EGF, was highly significant (P = 0.0032) indicating that EGFR expression was the determining factor for both the retention of BSD-EGF and the enhanced survival after BNCT.

**Dosimetry**. Dosimetric calculations were based on mean boron concentrations of tumor, brain, and blood at 24 h after i.t. injection of BSD-EGF and 2.5 h after i.v. administration of BPA. On the basis of total boron concentrations, the mean estimated physical radiation doses delivered to F98EGFR and F98WT tumors were calculated to be 11.0 and 7.3 Gy, respectively, after i.t. injection, 10.9 Gy with i.v. BPA alone, and 18.0 Gy in combination with i.t. BSD-EGF (Table 3). The normal brain doses ranged from 5.72 to 7.20 Gy. Compound biological effectiveness doses, which depend on the chemical form of the boron, could not be calculated because...

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**Table 1. Biodistribution of 125I-labeled BSD-EGF at 6 and 24 h after i.t. injection**

<table>
<thead>
<tr>
<th>Test groupa</th>
<th>Time (h)</th>
<th>Tumor</th>
<th>Brain (Ipsilateral)</th>
<th>Brain (Contralateral)</th>
<th>Liver</th>
<th>Blood</th>
<th>Tumor/brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>F98EGFR</td>
<td>6</td>
<td>68.5 ± 10.9</td>
<td>5.2 ± 1.2</td>
<td>1.2 ± 0.3</td>
<td>0.1 ± 0.1</td>
<td>0.05 ± 0.0</td>
<td>13.7 ± 2.5</td>
</tr>
<tr>
<td>F98EGFR</td>
<td>24</td>
<td>33.2 ± 6.6</td>
<td>9.1 ± 4.9</td>
<td>2.1 ± 1.4</td>
<td>0.9 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>3.5 ± 1.7</td>
</tr>
<tr>
<td>F98WT</td>
<td>6</td>
<td>64.5 ± 17.7</td>
<td>7.0 ± 1.8</td>
<td>1.6 ± 0.5</td>
<td>0.2 ± 0.1</td>
<td>0.09 ± 0.04</td>
<td>10.9 ± 3.0</td>
</tr>
<tr>
<td>F98WT</td>
<td>24</td>
<td>9.4 ± 1.0</td>
<td>7.1 ± 2.0</td>
<td>1.4 ± 0.8</td>
<td>1.0 ± 0.8</td>
<td>0.9 ± 0.3</td>
<td>1.9 ± 0.7</td>
</tr>
</tbody>
</table>

*Each received an i.t. injection of 5µCi of 125I-labeled BSD-EGF (40 µg boron 10µg EGF). Percentage of injected dose was based on the amount recovered relative to the amount administered.

This ratio is based on tumor uptake versus ipsilateral (tumor-bearing) cerebral hemisphere.
of the inability of DCP-AES to distinguish between boron in the form of BPA versus BSD-EGF.

**Brain Histopathology.** The brains of all of the rats were subjected to histopathologic examination. The tumor size indices at the time of death range from 2.6 to 2.7 for all groups of animals except F98EGFR-irradiated controls and animals that received i.v. BPA, which had slightly smaller tumors (tumor size index 2.3 and 1.9, respectively). Although it is not readily apparent why these animals died earlier than those with larger tumors, this has been observed by us in other BNCT studies (5,6), and it may have been because of an increase in cerebral edema. Microscopic examination revealed infiltration of adjacent white matter, tumor necrosis, and pseudopalisading similar to that described previously by us for the F98WT glioma (35). Necrosis was more prominent in large versus intermediate sized tumors and absent in small tumors. No acute radiation effects were seen, and because all of the animals died within 4 months of treatment, it was too early for the appearance of any late effects (36).

**DISCUSSION**

In the present study, we have shown that i.t. injection of BSD-EGF into F98EGFR glioma-bearing rats resulted in a tumor boron concentration that was 2.3 times greater than that of F98WT tumors. When i.t. injection of BSD-EGF was followed by BNCT, there was a significant increase in MST compared with that observed in animals bearing F98WT tumors of which the survival time was equivalent to that of F98EGFR-irradiated controls. These observations establish that there was specific molecular targeting of the EGFR, as indicated by both the biodistribution data and enhanced survival after BNCT. It is noteworthy that i.t. injection of BSD-EGF yielded survival data that were superior to those obtained after i.v. administration of BPA, which is believed to preferentially target metabolically active tumor cells (37,38), and yielded comparable survival data to those obtained with i.t. injection of BSD-EGF. Somewhat surprisingly, however, the combination of i.v. BPA and BSD-EGF did not result in a statistically significant increase in survival time over that obtained with i.t. BSD-EGF alone, and this was attributable to the wide range in survival times for animals in these two groups. It might well be that the time interval between administration of BPA and the initiation of BNCT was not optimum and that other time intervals will have to be investigated. However, there was a broadening of the range of survival times in rats that received the combination compared with that of rats that received BSD-EGF alone, and this suggests that if larger numbers of animals per experimental group had been used, the differences between the two groups might have attained statistical significance. A similar broadening of the range of survival times also has been observed by us in other studies using the F98 glioma model with BPA and BSD-EGF as the capture agents (5,6), suggesting that there were animal to animal variations in tumor boron content and its microdistribution. Data reported recently from both animal (39,40) and human studies (41,42) have shown that there were wide variations in the distribution of 10B within brain tumors after administration of either BPA or BSD-EGF. A more uniform microdistribution and higher concentrations of 10B within the tumor would result in a higher cell kill after BNCT (43), and either cure or significantly delay regrowth of the tumor.

We had developed previously a series of EGFR-transfected rat C6 glioma cell lines, designated C6EGFR, with glucocorticoid-inducible expression of the human EGFR gene (22). The C6EGFR glioma model initially was used by us to assess tumor uptake and retention of BSD-EGF after i.t. injection (10), and more recently after systemic administration (44). Tumor retention of radiiodinated BSD-EGF at 24 h after i.t. injection into C6EGFR glioma-bearing rats was 21.8% versus 68.5% ID/g in F98EGFR glioma-bearing rats compared with 5.6% in C6 (10) and 9.4% in F98 wild-type tumors. Despite the large differences in uptake of radiiodinated BSD-EGF in C6EGFR and F98EGFR glioma-bearing rats, somewhat unexpectedly, the corresponding values for tumor boron concentrations were relatively close to one another (15.2 versus 21.1 mg/g). One possible explanation for this may have been differences in the affinity constants (Kd) of the biocongjugates used in these two studies, which were carried out several years apart. Because only very small quantities of BSD-EGF localized in C6EGFR tumors after systemic administration of either 131I (10) or 99mTc-labeled BSD-EGF (44) (0.01 and 0.06% ID/g, respectively), the i.t. route was selected for administration of the BSD-EGF. Although the C6EGFR model has been useful for biodistribution studies, it is not suitable for studies to evaluate the efficacy

![Table 2: Boron concentrations and physical radiation doses delivered to tumor, brain, and blood](attached-image-url)

![Table 3: Survival times of F98 glioma-bearing rats after i.t. injection of BSD-EGF with or without i.v. BPA followed by BNCT](attached-image-url)
of BNCT because the parental tumor arose in an outbred Wistar rat (23), and there is no syngeneic host for this tumor. This is an especially important consideration for both gene therapy and BNCT, which can selectively result in the death of individual tumor cells and spare infiltrating host immune effector cells that potentially could mediate an alloimmune response. The F98EGFR glioma model, on the other hand, was derived from a tumor that arose in an inbred Fischer rat, and, therefore, can be propagated in a syngeneic host. Furthermore, it is only weakly immunogenic (45). Survival data of animals that received F98EGFR glioma cells intracerebrally were almost identical to those receiving F98WT cells, indicating that transfection of the gene encoding EGFR or surface membrane expression of human EGFR protein did not alter the tumorigenicity of the F98EGFR tumor.

In the present study we have used a transfected glioma cell line that uniformly expressed the wild-type EGFR. Because there is considerable variability in EGFR expression among malignant gliomas (11, 13, 14) and within individual tumors themselves (12), this receptor alone cannot be an effective target for gliomas in general or all of the constituent cells of an individual tumor. Therefore, other targeting strategies will be necessary. This could include antibodies (46–48) or peptides (49) that target a mutant form of EGFR, EGFRvIII (50), which has a more restricted expression on high-grade gliomas (51), as well as low molecular weight delivery agents such as BPA and BSH. Targeting the human EGFR (52), especially by MoAbs directed against the receptor, has recently become the subject of intense investigation (53–56). Promising results have been obtained using MoAb C225 in combination with either chemo- or radiotherapy (54, 55). Because wild-type EGFRs are expressed on a wide variety of normal tissues, especially the liver and kidneys (57, 58), the problems of nonspecific uptake and normal tissue toxicity of either MoAb- or EGF-based tumoricidal bioconjugates could be important. On the other hand, because BNCT is a binary system that requires, first, delivery of a sufficient amount of 10B, and second, neutron irradiation, which can be directed to a specific anatomical site at some later point in time, this would reduce or eliminate normal tissue toxicity. Furthermore, MoAbs directed against EGFR could potentially enhance radiation sensitivity of tumors (55, 56) and this may additionally augment the tumoricidal effects of BNCT.

Another major question that must be considered when using a high molecular weight boron-containing delivery agent for BNCT is whether the boronated ligand has a sufficiently high affinity and specificity for the receptor to permit in vivo cellular targeting. Bioconjugates produced by covalently coupling EGF and BSH to an allylated 70 kDa dextran chain had decreased specificity for EGFR as additional BSH groups were attached (21). In contrast, using BSA we have not seen a reduction in specificity of BSA-EGF, although the $K_A$ was reduced from $10^{-3}$ to $10^{-5}$ (9). Delivery of MoAb- or EGF-based bioconjugates to brain tumors is a particularly challenging problem because only small quantities can be expected to localize within the tumor after systemic administration (17–19). Although in the present study we have used direct i.t. injection, more recently we have used convection enhanced delivery (59, 60) to improve tumor uptake of both BSD-EGF (61) and a MoAb, L8A4, directed against EGFRvIII (62). CED can potentially improve the delivery of both low and high molecular weight agents both to the brain and brain tumors by applying a pressure gradient to establish bulk flow during interstitial inflation after which diffusion can occur. CED of BSD-EGF resulted in a 7.3 times increase in the volume of distribution within the infused cerebral hemisphere and a 1.8 times increase in tumor uptake of BSD-EGF compared with i.t. injection (60). Future studies using either BSD-EGF or boronated MoAb L8A4 for BNCT will use CED.

In conclusion, the present study has demonstrated that a high molecular weight boron-containing delivery agent, BSD-EGF, could specifically target receptor-positive tumor cells in vivo and produce an increase in survival time after BNCT. The present study is paradigmatic for future studies using high molecular weight, receptor-mediated, tumor targeting agents such as EGF, or other growth factor-based bioconjugates or MoAbs. Furthermore, it provides a basis for the future development of high molecular weight agents for BNCT either alone or in combination with low molecular weight agents.

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Molecular Targeting of the Epidermal Growth Factor Receptor for Neutron Capture Therapy of Gliomas

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