Intratumoral Delivery of Boronated Epidermal Growth Factor for Neutron Capture Therapy of Brain Tumors

Weilian Yang, Rolf F. Barth, Dianne M. Adams, and Albert H. Soloway

Department of Pathology [W. Y., R. F. B., D. M. A.] and College of Pharmacy [A. H. S.], The Ohio State University, Columbus, Ohio 43210

ABSTRACT

The gene for epidermal growth factor receptor (EGFR) is amplified or overexpressed in high-grade gliomas but is low or undetectable in normal brain. Recently, there has been increasing interest in using epidermal growth factor (EGF)-based bioconjugates as targeting agents for brain tumors. In the present study, we have investigated the potential use of boronated EGF as a delivery agent for boron neutron capture therapy, which is based on the capture reaction that occurs when ^10^B, a stable isotope, is irradiated with low-energy thermal neutrons. A fourth generation starburst dendrimer was boronated and linked to EGF using heterobifunctional reagents. Either wild-type or EGFR gene transduced C6 glioma cells (C6EGFR), which expressed 10^10 receptor sites/cell, were stereotactically implanted into the right cerebral hemisphere of Fischer rats. Four weeks later, the rats received either i.v. or intratumoral (i.t.) injection of ^125^I-labeled boronated starburst dendrimer (BSD) or BSD-EGF. The biodistribution of ^125^I-BSD-EGF and ^125^I-BSD was studied by means of whole-body scintigraphy, autoradiography, and gamma scintillation counting. Following i.t. injection of ^125^I-BSD-EGF, 21.8% of the injected dose per gram tissue (% ID/g) was localized in C6EGFR tumors at 24 h and 16.3% at 48 h compared to 5 and 1.3% ID/g in C6 wild-type tumors, respectively, and 0.01 and 0.096% ID/g, respectively, for i.v. injected animals at the corresponding times. In contrast, following i.t. injection of BSD-EGF, only 0.01—0.1% ID/g was localized in the liver and spleen at 24 and 48 h compared to 5—12% ID/g following i.v. injection. Our data indicate that direct i.t. injection can selectively deliver BSD-EGF to EGFR-positive gliomas and suggest that intracerebral administration may be the most effective way for delivering EGF-based bioconjugates to EGFR-positive brain tumors.

INTRODUCTION

BNCT is a binary system based on the selective uptake of sufficient amounts (~10^9 atoms/cell) of a stable isotope, ^10^B, by tumor cells, followed by irradiation with low energy (<0.025 eV) thermal neutrons. The resulting nuclear capture and fission reactions yield a-particles and 7Li nuclei, which have high linear energy transfer and sufficient range to cause high doses of radiation to the tumor while sparing normal tissues. These requirements are discussed in detail in several recent reviews and monographs (1—3). One of the major challenges in treating high-grade brain tumors with BNCT is how to deliver a sufficient amount of ^10^B to individual tumor cells to sustain a lethal ^10^B(n,a)7Li capture reaction.

Using a combination of BBB disruption and intracarotid injection of either sodium borocaptate or boronophenylalanine, we have been able to significantly enhance both the delivery and therapeutic efficacy of these low molecular weight agents using the F98 rat glioma model (4). Their uptake by tumor cells, however, is not receptor or epitope dependent, and for this reason, we have been interested in the possibility of using monoclonal or bispecific antibodies (5, 6) and boronated EGF (7) as specific targeting agents either alone or, more likely, in combination with low molecular weight boron compounds to target different subpopulations of tumor cells. The EGF gene often is amplified in human glioblastomas and other primary brain tumors, but it is low or undetectable in normal brain (8). Studies by Bigner et al. (9) revealed that in a series of 33 human glioma biopsies, 15 showed amplification of the EGF gene. Similar or even higher frequencies of amplification have been observed by others, and this often is associated with increased cell surface receptor expression (10, 11). The distribution of EGF in high-grade gliomas is variable, which probably reflects the cellular heterogeneity of these tumors. Because the number of EGFRs on individual tumor cells can be 100 times greater than that on normal glial cells (12), the EGF receptor has been considered as a potential target for the specific delivery of diagnostic and therapeutic agents to brain tumors (13—20). Initially, the focus was on using anti-EGFR monoclonal antibodies or their fragments (13, 14), but because such small amounts (0.0001 to 0.00001%) of the injected dose are localized in brain tumors following systemic injection (15, 16), it seems unlikely that this approach will be successful. One alternative is to use EGF itself as the delivery agent, and there have been a number of recent reports describing the use of either ^125^I or boronated EGF bioconjugates as potential targeting agents for brain tumors (7, 17—20). The advantage of EGF-based bioconjugates include: (a) lower molecular weight—than antibody-based conjugates; (b) more favorable diffusion properties within brain and tumor; (c) receptor-mediated binding with endocytosis of the bioconjugate; and (d) regeneration of the EGF.

Recently, we have described a method for the boronation of EGF and have characterized the resulting bioconjugates in vitro (17). One major problem associated with the use of EGF as a targeting agent was that very large amounts of the injected bioconjugate localized in the liver following systemic injection. To obviate this problem, we initially studied the localization of ^99^mTc-labeled EGF (20) following intracerebral injection into rats bearing implants of the C6 glioma, which had been transfected with the gene encoding EGF (21). These studies showed that EGF preferentially was retained in C6EGFR tumors (20) and suggested that i.t. injection might be useful for the delivery of EGF-based bioconjugates to EGFR-positive brain tumors. In the present study, we have investigated the potential usefulness of boronated EGF as a delivery agent for BNCT in rats bearing intracerebral implants of the C6EGFR glioma. As described in detail in the following report, effective targeting of gliomas could be achieved following i.t. injection of boronated EGF, and the amount of boron...
delivered to these tumors exceeded by 3–4 orders of magnitude that which could be delivered by i.v. injection.

**MATERIALS AND METHODS**

Preparation and Purification of Boronated EGF Bioconjugate. A four-generation “starburst” dendrimer, which was composed of repetitive polyamido amino groups arranged in a starburst pattern, was boronated with a methylsilycanato polyhedral borane anion, Na[CH3]3NBH1@H3NCO, to yield BSD using a procedure we recently described elsewhere (17). The BSD was reacted with N-succinimidyl 3-(2-pyridyldithio)propionate, and the resulting product was cleaved with DTT to yield a sulphydryl-containing BSD. EGF was derivatized with the heterobifunctional reagent m-maleimidobenzoyl-N-hy-

droxsuflousuccinimide ester and linked to sulphydryl-containing BSD to yield a BSD-EGF bioconjugate (17). The bioconjugate was purified by column chromatography using a Sephadex-G50 column and eluted with 0.1 m Tris and 0.2 m NaCl buffer (pH 8.5). One-mI fractions were collected, and protein concentrations were determined spectrophotometrically by measuring absorbance at 280 nm using a Beckman DU-6 spectrophotometer (Beckman Instrument Inc., Irvine, CA). Boron was quantified by means of DCP-AES using a Spectraspan VB spectrometer (Applied Research Laboratories, La Brea, CA), as described in detail by Barth et al. (22). Fractions containing peak concentrations of both protein and boron were pooled and used in the studies described in the following section.

**In Vitro Receptor Binding and Boron Uptake of BSD-EGF.** Rat C6 wild-type glioma cells (American Type Culture Collection, Rockville, MD) and C6EGF cells, which had been transfected with the human gene encoding EGFFR to yield stable transfectants (21), were used in the studies described below. All cells were grown in DMEM containing glucose, l-glutamine, and 10% fetal bovine serum. Receptor binding activities of boronated and native EGF with C6EGF cells were studied by means of a competitive binding assay, as described in detail by us (17, 21) elsewhere. Briefly, C6EGF cells (2 × 106 cells/well) were seeded into 24-well flat-bottomed plates (Coming, Inc., Corning, NY) and allowed to attach overnight. Medium was replaced with DMEM containing 1 mm dexamethasone (Sigma Chemical Co., St. Louis, MO) and incubated overnight at 37°C to increase expression of EGFR (21). This was replaced with serum-free DMEM and incubated for 2 h, following which the cells were washed with PBS (pH 7.2). Varying concentrations (0–500 nm) of both native and boronated EGF were added to duplicate wells and incubated at ambient temperature in an atmosphere containing 5% CO2 for 2 h. The cells then were washed, and medium containing 0.18 nm 125I-EGF was added. Cells were incubated for an additional 2 h at ambient temperature, washed three times with PBS, and then harvested using 0.5 mm EDTA in PBS; cell-associated radioactivity was determined by γ scintillation counting (model 1185; Trum Analytic, Elk Grove Village, IL).

As determined by a radioligand binding assay and Scatchard analysis, wild-type C6 cells did not express EGFFR, whereas C6EGF expressed 3 × 106 EGFFR/cell (21). To determine if BSD-EGF retained its mitogenic effect on C6EGF glioma cells, a cell proliferation assay was performed. C6EGF cells (106/well) were dispersed in a 96-well, flat-bottomed plate and allowed to grow for 2 days. Then, increasing concentrations of native EGF or BSD-EGF (0–5000 nm) and 0.2 μCi [3H]thymidine (Amersham Corp., Arlington Heights, IL; 5 Ci/mmol) were added to each well and incubated for 6 h at 37°C, following which the cells were harvested and counted in a Packard Topcount micropulse scintillation counter (Packard Instrument Co., Inc., Meriden, CT). To determine the cellular uptake of BSD-EGF, 2.5 mg BSD-EGFR (~1000 boron atoms/EGF molecule) were added to each of four T150 tissue culture flasks (Coming, Inc.) containing either C6 wild-type or C6EGF cells, which had been grown to confluency. These were incubated for 1 h at 4°C, following which the cells were washed, harvested, and counted in a hemocytometer; boron concentrations were determined by DCP-AES (22). Boron values were expressed as micrograms per gram weight (i.e., per 106 cells) by multiplying by the appropriate factors.

**Radiolodination of BSD.** BSD and the BSD-EGF bioconjugates were reacted with Bolton-Hunter reagent to introduce a phenolic function into these structures (23). Briefly, a 10-fold molar excess of Bolton-Hunter reagent (Pierce Chemical Co., Rockford, IL) was added to BSD or BSD-EGF and cooled on ice for 1 h; following that procedure, unreacted reagent was removed using a Bio-Spin P-6 column (Bio-Rad Laboratories, Hercules, CA). BSD-EGF and BSD were then radioiodinated with 131I-NaI by means of the procedure described by us (17) using chloramine-T (2 mg/ml in 0.5 m phosphate buffer, pH 7.5; ICN Biomedicals Inc., Costa Mesa, CA). 131I-labeled BSD or BSD-EGF were shown to be stable and were not dehalogenated for at least 1 week when kept at 4°C.

**Animal Model and In Vivo Studies.** C6 wild-type or C6EGF glioma cells (106) were stereotactically implanted into the caudate nucleus of the right cerebral hemisphere of CD-Fischer rats (Charles River Laboratories, Wilmington, MA) using a procedure described previously (24). Briefly, rats were sedated by i.p. administration of a 1:2.1 mixture of ketamine/xylazine (9 mg/kg weight, after which a plastic screw (Arrow Machine Manufacturing, Inc., Richmond, VA) 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 105 cells/10 μl of serum-free DMEM containing 1.5% 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 weighed three times per week following tumor implantation. Twenty-five to 28 days following implantation, when signs (weight loss, lethargy, hunching, and ataxia) of a progressively growing intracerebral tumor were evident, rats were divided into four experimental groups consisting of 15–18 animals each. Groups 1–3 had C6EGF tumors, and group 4 had C6 wild-type tumors. Groups 1 and 4 received i.t. injection of 131I-labeled BSD-EGF (5 μCi/10 μg of EGF); group 2 received i.v. injection of 131I-labeled BSD-EGF; and group 3 received i.t. 131I-labeled BSD (5 μCi). Intratumoral injection was performed through the central hole in the plastic screw, which was embedded at the time of tumor implantation. All test agents (10 μl volumes) were injected over 2 min by means of a 50-μl Hamilton syringe with a 27-gauge needle equipped with a plastic collar to restrict the depth of penetration to the same level as that used for tumor implantation.

**Biodistribution Studies.** The biodistribution of 131I-BSD-EGF and 131I-BSD was studied at 1, 6, 24, and 48 h after injection. Animals were euthanized by an overdose of halothane. Tumors and normal tissues were collected and weighed, and tissue and organ uptake of 131I were determined by means of gamma scintillation counting using a well counter. Each tissue sample was counted together with triplicate samples of the injectate to correct for the decay of isotope before gamma counting. Boron concentrations in tumor and normal tissues were determined by means of DCP-AES (22). The Wilcoxon Gehan rank sum two-sample test was applied to the biodistribution data to test for significant differences between groups.

**Scintigraphic Studies.** Rats bearing either C6 wild-type or C6EGF gliomas were injected i.t. with 131I-BSD-EGF (40 μCi/80 μg of EGF). The distribution of radioactivity was monitored by means of external gamma scintigraphy using a Technicare 438 gamma camera (25), which was interfaced with a Technicare 560 computer (Ohio Nuclear, Inc., Solon, OH) for imaging data acquisition and processing. The amount of 131I radioactivity retained in the tumor was determined as a function of time using intrinsic features of the computer software, which permitted calculation of the average number of counts/pixel in each outlined region of interest.

** Autoradiography.** Brains of some animals, which had been euthanized at 24 h following i.t. injection of 131I-BSD-EGF, were removed, frozen in isopentane (2-methylbutane), which had been cooled to approximately −150°C in liquid nitrogen, and stored at −70°C until sectioning. Brains were mounted on chucks and cut coronally at 6–10-μm thickness on a cryostat (Miles Scientific, Naperville IL). Macroautoradiographs were made by placing the slides, which had a single coronal section of brain, in direct contact with Kodak XAR-5 stripping film (Eastman Kodak, Co., Rochester, NY) and exposing them for 8 days at 4°C in X-ray cassettes with intensifying screens. For micro-autoradiography, the tissue sections were coated with NTB2 dipping emulsion (Eastman Kodak), and stored at 4°C in light-proof boxes for 3 weeks, following which they were developed with Kodak D19 developer (26). Sections were counterstained with H&E for histological study.

**RESULTS**

**In Vitro Biological Properties of BSD-EGF.** As described by a competitive binding assay (Fig. 1), approximately three to four times...
the amount of EGF-BSD compared to native EGF was required to produce a 95% reduction in the binding of $^{131I}$-EGF to C6EGFR cells. By means of Scatchard analysis of these data, it was determined that native EGF and BSD-EGF recognized both high- and low-affinity receptors. Affinity constants ($K_a$) and quantitation of receptor sites for C6EGFR cells are summarized in Table 1. The $K_a$ of native EGF for high affinity receptors was $\sim$13 times greater than that of BSD-EGF, and the corresponding number of EGFR detected by EGF was 5.9 times greater than those detected using BSD-EGF. Based on the data shown in Fig. 1, iodinated BSD-EGF retained $\sim$90% of its in vitro affinity for EGFR compared to native EGF. As determined by the uptake of $[^3H]$thymidine, BSD-EGF and native EGF were weakly mitogenic for C6EGFR cells (Fig. 2) but, as expected, had no effects on C6 wild-type cells (data not shown). The in vitro uptake of boron by C6EGFR cells (Fig. 2) but, as expected, had no effects on C6 wild-type cells (data shown). The in vitro uptake of boron by C6EGFR cells following exposure to BSD-EGF was four times greater (10.2 ± 0.5 μg/10$^9$ cells) than that of C6 wild-type cells (2.6 ± 0.5 μg/10$^9$ cells), which did not express EGFR. The latter number probably represents nonspecific uptake, possibly due to an interaction between the protonated BSD moiety and the target cell surface membrane.

**Intracerebral C6EGFR Tumor Model.** After intracerebral implantation of 10$^8$ C6EGFR cells, all animals died within 26–34 days (21). At the time that the study was initiated, animals were randomized according to weight. In vivo localization studies were conducted 25–28 days after intracerebral implantation, at which time there were no statistically significant differences (Wilcoxon-Gehan rank sum test, $P = 0.1$) in mean tumor weights (0.16 ± 0.05, 0.15 ± 0.06, 0.17 ± 0.05, and 0.14 ± 0.08 g) among animals from groups 1–4. Although the tumors appeared to be well circumscribed macroscopically, microscopic examination revealed infiltration of adjacent white matter, tumor necrosis, and pseudopalisading (21).

**Table 1.** Affinity constants ($K_a$) and the numbers of receptor sites for C6EGFR cells$^a$

<table>
<thead>
<tr>
<th></th>
<th>High</th>
<th>Low</th>
<th>EGFR/cell</th>
<th>High</th>
<th>Low</th>
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</thead>
<tbody>
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<td>EGF</td>
<td>$6.93 \pm 3 \times 10^8$</td>
<td>$1.33 \pm 0.02 \times 10^6$</td>
<td>$8.35 \pm 5.6 \times 10^6$</td>
<td>$0.35 \pm 3.5 \times 10^5$</td>
<td>$7.63 \pm 7.1 \times 10^4$</td>
</tr>
<tr>
<td>BSD-EGF</td>
<td>$5.26 \pm 4.8 \times 10^{-9}$</td>
<td>$9.89 \pm 0.59 \times 10^{-7}$</td>
<td>$0.14 \pm 1.5 \times 10^{-6}$</td>
<td>$5.3 \pm 5.8 \times 10^{-7}$</td>
<td>$3.8 \pm 1.8 \times 10^{-7}$</td>
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$^a$ Affinity constants and numbers of receptor sites were determined by Scatchard analysis. EGFR could not be detected on C6 wild-type cells.

**Biodistribution Studies.** The biodistribution profiles of $^{131I}$-BSD-EGF following i.v or i.t injection into C6EGFR glioma-bearing rats are shown in Fig. 3. Both the expression of EGFR and the route of administration (i.t. versus i.v.) were important in determining localization and persistence of $^{131I}$-labeled BSD-EGF. Following i.t. injection of $^{131I}$-BSD-EGF (Fig. 3A), 21.8% of the ID/g tissue was localized in C6EGFR tumors at 24 h and 16.3% at 48 h compared to 0.01 and 0.006% ID/g ($P < 0.002$), respectively, for animals receiving i.v. injections (Fig. 3B). In contrast, following i.t. injection of BSD-EGF, only 0.01–0.1% ID/g was localized in the liver and spleen at 24 and 48 h compared to 5–12% ID/g ($P < 0.002$) following i.v. injection. Tumor uptake of radioactivity following i.t. injection of $^{131I}$-BSD or $^{131I}$-BSD-EGF in rats bearing C6 or C6EGFR gliomas is summarized in Fig. 4. Between 1 and 6 h following i.t. injection, 40–70% ID/g of BSD-EGF was localized in C6EGFR and C6 wild-type gliomas, and the differences between the two groups were not statistically significant ($P > 0.1$). By 24 and 48 h after injection, however, the amount of $^{131I}$-BSD-EGF in C6 wild-type gliomas had declined to 5.6 and 1.3% ID/g, respectively, compared to 21.8 and 16.3% ID/g for C6EGFR gliomas ($P < 0.002$). Enhanced tumor uptake and persistence of the BSD-EGF bioconjugate in C6EGFR tumors was specifically determined by the EGF molecule, because only 0.3% ID/g of BSD was detected in the liver and spleen at 24 and 48 h following injection of $^{131I}$-BSD into C6 or C6EGFR gliomas. The biodistribution profiles of the radiolabeled bioconjugates at 24 h following different routes of administration to C6 and C6EGFR glioma-bearing rats are summarized in Table 2. The highest uptake was found in C6EGFR gliomas following i.t. injection of $^{131I}$-BSD. The tumor boron concentration was $15.3 \pm 5.2 \mu g/g$ (44% ID) at 24 h following i.t. injection of BSD-EGF into C6EGFR glioma, whereas boron concentrations in normal brain, blood, liver, kidney, and spleen were all nondetectable (i.e., $<0.5 \mu g/g$).

**Autoradiographic Studies.** Autoradiographs were made from brains of C6EGFR and C6 wild-type glioma-bearing rats that had been euthanized at 24 h after i.t. injection of $^{131I}$-BSD-EGF. Whole-brain autoradiographs revealed that by 24 h after injection, $^{131I}$-BSD-EGF had accumulated and diffused into C6EGFR tumors and the brain surrounding the tumor (Fig. 5), whereas in contrast, there was no evidence of accumulation in C6 wild-type tumors. Although grain densities varied in different areas of the same tumor, microautoradio-

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**Fig. 1.** Competitive binding assay using $^{125I}$-EGF. C6EGFR cells ($\sim$10$^9$ cells/well) were incubated with medium containing 0.185 nM of $^{125I}$-EGF and varying concentrations (0–500 nM) of either native EGF or BSD-EGF at ambient temperature for 2 h. Following which cell-associated radioactivity was determined by gamma scintillation counting. Each point represents the mean of six replicates ± 15%.

**Fig. 2.** Mitogenic activity of EGF and BSD-EGF. C6EGFR cells ($\sim$10$^9$ cells/well) were grown in 96-well plates for 48 h, following which they were incubated at 37°C with $[^3H]$thymidine (1 μCi/ml) and increasing concentrations of native or BSD-EGF (0–5000 nM) EGF for an additional 6 h. The cells then were harvested, and cell-associated $^3$H radioactivity was determined by β-scintillation counting. Each point represents the mean of six replicates ± 15%.
hepatic uptake (0.01–0.1%) of BSD-EGF after i.t. injection of BSD-EGF into C6\textsubscript{EGFR} glioma-bearing rats. In contrast to this, there was low tumor uptake (0.01 and 0.006%, respectively) and high hepatic and splenic uptake (5–12%) at 24 and 48 h following i.v. administration of BSD-EGF, indicating that both the route of administration and EGFR expression were important determinants for tumor uptake. The major problem associated with systemic i.v. delivery of high molecular weight agents, such as monoclonal antibodies, to tumors is that only an exceedingly small fraction of the injected dose (0.001–0.01% ID/g) is localized within the tumors (27, 28), and even smaller amounts of antibody are delivered to brain tumors (16, 29) because of the BBB (30). In a recent Phase I study to evaluate the possible use of a monoclonal antibody directed against EGFR to target human gliomas, the amount of antibody that reached the tumor following i.v. injection was in the range of 0.001–0.0001% ID/g (16). These observations strongly suggest that it is highly unlikely that i.v. injection alone will be effective for delivering high molecular weight agents to brain tumors, and that other approaches, such as direct i.t. injection or interstitial convection enhanced delivery, will be required (31–37).

In contrast to the low tumor uptake following i.v. administration of BSD-EGF, large amounts of BSD-EGF accumulated in the two major organs of the reticuloendothelial system, the liver and spleen (12.8 and 5.4% ID/g, respectively). The high hepatic uptake of BSD-EGF, in part, may be related to the propensity of both BSD and EGF to localize in this organ. In previous studies, we have shown that non-boronated starburst dendrimers localized in the liver and spleen, and that the amounts appeared to be directly related to the molecular weight and number of reactive terminal amino groups of the dendrimer (4). Gedda et al. (19) have reported that hepatic and renal uptake of \textsuperscript{125I}-EGF-dextran-sodium borocaptate was high following i.v. administration. We have observed by means of external gamma scintigraphy, that following i.v. administration of \textsuperscript{99m}Tc-EGF to

\begin{table}
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\begin{tabular}{lcccccccc}
\hline
Glioma type & Test agent & Route & Liver & Spleen & Blood & Ipsilateral & Contralateral & Tumor \\
\hline
C6\textsubscript{EGFR} & BSD-EGF & i.t. & 0.1 ± 0.07 & 0.2 ± 0.09 & 0.08 ± 0.03 & 1.4 ± 0.5 & 0.7 ± 0.5 & 21.8 ± 3.7 \\
C6\textsubscript{EGFR} & BSD-EGF & i.v. & 0.04 ± 0.003 & 0.007 ± 0.004 & 0.02 ± 0.004 & 0.01 ± 0.01 & 0.007 ± 0.003 & 0.3 ± 0.2 \\
C6\textsubscript{EGFR} & BSD & i.t. & 0.01 ± 0.005 & 0.01 ± 0.004 & 0.02 ± 0.004 & 0.01 ± 0.01 & 0.007 ± 0.003 & 0.3 ± 0.2 \\
C6\textsubscript{EGFR} & BSD-EGF & i.v. & 12.8 ± 0.9 & 5.4 ± 2.4 & 0.08 ± 0.01 & 0.004 ± 0.002 & 0.004 ± 0.002 & 0.01 ± 0.003 \\
\hline
\end{tabular}
\caption{Biodistribution profiles of \textsuperscript{131I}-labeled BSD-EGF and BSD in glioma-bearing rats\textsuperscript{a}}
\end{table}

\textsuperscript{a} \textsuperscript{131I}-BSD-EGF (5 μCi/10 μg of EGF) was administered i.t. or i.v., and animals were killed 24 h later. Radioactivity was determined by gamma scintillation counting. Tissue uptake is expressed as % ID/g tissue, and each value represents the arithmetic mean ± SD of four to seven rats.

DISCUSSION

In the present study, we have shown that there was high tumor uptake at 24 and 48 h (21.8 and 16.5% ID/g, respectively) and low hepatic uptake (0.01–0.1%) of BSD-EGF after i.t. injection of BSD-EGF into C6\textsubscript{EGFR} glioma-bearing rats. In contrast to this, there was low tumor uptake (0.01 and 0.006%, respectively) and high hepatic and splenic uptake (5–12%) at 24 and 48 h following i.v. administration of BSD-EGF, indicating that both the route of administration and EGFR expression were important determinants for tumor uptake. The major problem associated with systemic i.v. delivery of high molecular weight agents, such as monoclonal antibodies, to tumors is that only an exceedingly small fraction of the injected dose (0.001–0.01% ID/g) is localized within the tumors (27, 28), and even smaller amounts of antibody are delivered to brain tumors (16, 29) because of the BBB (30). In a recent Phase I study to evaluate the possible use of a monoclonal antibody directed against EGFR to target human gliomas, the amount of antibody that reached the tumor following i.v. injection was in the range of 0.001–0.0001% ID/g (16). These observations strongly suggest that it is highly unlikely that i.v. injection alone will be effective for delivering high molecular weight agents to brain tumors, and that other approaches, such as direct i.t. injection or interstitial convection enhanced delivery, will be required (31–37).

In contrast to the low tumor uptake following i.v. administration of BSD-EGF, large amounts of BSD-EGF accumulated in the two major organs of the reticuloendothelial system, the liver and spleen (12.8 and 5.4% ID/g, respectively). The high hepatic uptake of BSD-EGF, in part, may be related to the propensity of both BSD and EGF to localize in this organ. In previous studies, we have shown that non-boronated starburst dendrimers localized in the liver and spleen, and that the amounts appeared to be directly related to the molecular weight and number of reactive terminal amino groups of the dendrimer (4). Gedda et al. (19) have reported that hepatic and renal uptake of \textsuperscript{125I}-EGF-dextran-sodium borocaptate was high following i.v. administration. We have observed by means of external gamma scintigraphy, that following i.v. administration of \textsuperscript{99m}Tc-EGF to...
Fig. 5. Representative brain section and macroautoradiograph of a C6EGFR glioma-bearing rat. Animals were injected i.t. with 5 μCi of 131I-BSD-EGF and killed 24 h later. A coronal section stained with H&E (A) and a representative autoradiograph (B) revealed that radioactivity accumulated in the tumor and the brain surrounding the tumor.

C6EGFR glioma-bearing rats, large amounts (5–30% ID/g) of 99mTc-EGF were localized in the liver, spleen, and kidneys, compared to small amounts in brain tumor (0.05–0.1% ID/g) (20). The high propensity of EGF to localize in the liver and kidneys (25) following systemic administration has limited the clinical use of EGF-based bioconjugates as a targeting agents for tumors, in general, and more specifically for brain tumors. Intratumoral injection of EGF has circumvented the most serious problems associated with systemic delivery of EGF bioconjugates, i.e., low tumor and high hepatic uptake.

One of the major advantages of receptor-targeted delivery of a tumoricidal agent is the potential for high specificity (12). Although our data revealed no significant differences in tumor uptake by C6 wild-type and C6EGFR between 1–6 h following i.t. administration, BSD-EGF was more rapidly cleared from wild-type C6 gliomas. By 24 h, the amount of BSD-EGF retained in EGFR-positive tumors was 4–10 times greater than EGFR-negative tumors and 60 times greater than the nonspecific retention of BSD in EGFR-positive tumors. Based on these observations, the optimum time to initiate BNCT following i.t. injection of BSD-EGF probably would be at 24 h, at which time normal brain boron levels would be low, but tumor boron levels would be in the therapeutic range (i.e., >15 μg/g tumor). External gamma scintigraphy and autoradiography also demonstrated that 131I-BSD-EGF was retained in C6EGFR but not in C6 wild-type gliomas at 24 and 48 h following i.t. injection of 131I-BSD-EGF, indicating that tumor uptake and retention of the bioconjugate was dependent upon EGFR expression.

Delivery of both low and high molecular weight agents to brain tumors presents a special problem because of the BBB, which impedes the delivery of both water-soluble drugs and high molecular weight bioconjugates to this anatomical site (30, 38). Therefore, interstitial delivery, which bypasses the BBB, is being investigated as a means of improving drug delivery to both the brain and intracerebral tumors (36, 37, 39). Regional delivery is particularly well suited for brain tumors, because more than 90% of malignant gliomas recur within 2 cm of the original margins of resection (40), and metastases outside of the central nervous system are rare (41). Local control of glioma potentially can prevent or delay tumor recurrence and prolong disease-free survival (42). Brain tumor resection may be advantageous for regional therapy, because the resulting surgical void space can be loaded with drugs that otherwise would not traverse the BBB (42). Interstitial radiation therapy has had some success in controlling gliomas (34). Interstitial chemotherapy (43–45) and monoclonal antibody-targeted radiotherapy (46–48), which are more selective and less neurotoxic, presently are being evaluated for the treatment of patients with primary and metastatic brain tumors. BNCT theoretically is a highly specific modality for treating brain tumors, if the boron containing agent can be selectively targeted to glioma cells that remain following surgical resection of the main tumor mass.

The present study is the first to demonstrate that boronated EGF potentially can be used in vivo as a delivery agent for BNCT of brain tumors by using the i.t. route of administration. The amount of boron required to sustain a lethal 10B(n,α)7Li capture reaction for BNCT is in the range 15–30 μg/g (1). Following a single i.t injection of BSD-EGF (34 μg of boron/10 μg of EGF), 44% ID/g of boron was retained in C6EGFR gliomas, and the tumor boron concentration was 15.2 ± 5.2 μg/g at 24 h, which was within the therapeutic range. For receptor-mediated targeting to be successful, there must not only be delivery of sufficient amounts of 10B to the tumor but also relatively uniform macro and micro distributions of 10B to individual tumor cells (49). We have shown by means of autoradiography that following a single i.t. injection, BSD-EGF diffused into the tumor and the brain tissue surrounding the tumor but did not reach all cells of the tumor. For this reason, we believe that a combination of both low and high molecular weight boron delivery agents would most effectively...
target the heterogeneous population of cells that are found within gliomas. The volume of distribution ($V_d$) in tissue that can be attained by direct interstitial infusion of therapeutic agents into the brain without adverse effects (37, 51). By using convection to deliver of large molecules ($M$, 126,000) to significant regions of the brain without adverse effects (37, 51). By using convection to supplement simple diffusion, the delivery of drugs can be significantly increased within the brain, and this may be directly relevant to the delivery of BSD-EGF to gliomas. Interstitial delivery of drugs to brain tumors also can be enhanced by means of drug-impregnated polymers, which provide sustained drug release (35), or implantable osmotic pumps (36), which produce a convection impregnated polymers, which provide sustained drug release (35), or implantable osmotic pumps (36), which produce a convection gradient within the brain (39, 43—45).

The rat C6 glioma and the C6EGF transfectants, which were used in the present study, were a good model for investigating the localization and in vivo retention of BSD-EGF. However, because the C6 glioma arose in an outbred Wistar rat and it is allogeneic to all inbred strains, it would not be a good model to use to assess the therapeutic efficacy of BSD-EGF as an in vivo targeting agent for BNCT (52), unless it were implanted into the brains of nude rats, which are incapable of mounting an alloimmune response. Under the appropriate circumstances, the C6 glioma can evoke an intense intracerebral immune response (53). To obviate this, Fenstermaker and coworkers (20), using the same vector construct that was used to produce the C6EGF transfectants, recently has transfected the EGF gene into the weakly immunogenic F98 glioma (54), which we have used extensively in our studies for BNCT (55, 56). Preliminary studies indicate that F98-transfected EGF glioma cells constitutively express EGF but at much lower levels than C6EGF cells.

In conclusion, in the present study we have demonstrated the feasibility of using direct i.t. administration of boronated EGF to deliver $^{10}B$ specifically to EGFR-positive brain tumors for BNCT. Studies on the use of convection-enhanced delivery and osmotic pumps to improve the delivery of BSD-EGF to EGFR-positive rat brain tumors for BNCT are presently in the planning stage in our laboratory.
INTRATUMORAL DELIVERY OF BORONATED EGF


Intratumoral Delivery of Boronated Epidermal Growth Factor for Neutron Capture Therapy of Brain Tumors

Weilian Yang, Rolf F. Barth, Dianne M. Adams, et al.


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