Development of a Syngeneic Rat Brain Tumor Model Expressing EGFRvIII and Its Use for Molecular Targeting Studies with Monoclonal Antibody L8A4

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ABSTRACT

Purpose: The goals of the present study were 2-fold: (a) to develop and characterize a rat brain tumor model that could be used for studies of molecular targeting of EGFRvIII and (b) to study the tumor localizing properties of radio-labeled monoclonal antibody (mAb) L8A4, specifically directed against EGFRvIII, following systemic, i.t., and convection enhanced delivery to brain tumor–bearing rats.

Experimental Design and Results: F98 wild-type (F98WT) rat glioma cells were transfected with a gene encoding human EGFRvIII, and following selection and cloning, a cell line, designated F98npEGFRvIII, was identified, which expressed a nonconstitutively phosphorylated form of the receptor. As determined by a radioligand binding assay, there were 1.2 \times 10^5 EGFRvIII sites per cell compared with an undetectable number on F98WT cells. The tumorigenicity of the F98npEGFRvIII glioma was studied following i.c. implantation of 10^7, 10^8, or 10^9 cells into CD-Fischer rats. Mean survival times were 23, 17, and 13 days, respectively, which were equivalent to those obtained with F98EGFR and F98WT cells. As determined by magnetic resonance imaging, the mean doubling times for the F98WT and F98npEGFRvIII gliomas were similar (59.8 ± 4.8 versus 52 ± 3.3 hours). Following i.v. administration to glioma-bearing rats, mAb L8A4 specifically targeted the F98npEGFRvIII glioma, and at 24 hours, 7.7% of the injected dose per gram (ID/g) localized in the tumor. This increased 5-fold to 39.5% ID/g following i.t. injection and 7-fold to 59.8% ID/g at 24 hours following convection enhanced delivery.

Conclusions: Based on these data, we have concluded that the F98npEGFRvIII glioma should be a valuable animal model for therapy studies focusing on molecular targeting of EGFRvIII by receptor specific mAbs.

INTRODUCTION

The gene encoding epidermal growth factor (EGF) receptor (EGFR) often is amplified and mutated in human gliomas, but expression is low or undetectable in normal brain (1–3). Based on this, EGFR and its mutant isoform, variant III of the human EGFR (EGFRvIII), are under intensive investigation as potential molecular targets for the specific delivery of diagnostic and therapeutic agents to brain tumors (4–7). We have been interested in the possibility of using either EGF itself (8–13) or anti-EGFR monoclonal antibodies (mAb; ref. 14) as 10B delivery agents for neutron capture therapy (NCT; ref. 13) of high-grade gliomas. To fully evaluate this approach, we initially produced a series of human EGFR-expressing transfectants of the rat C6 glioma (15). However, because this tumor originated in an outbred Wistar rat and has no syngeneic host (13), it can evoke an alloimmune response that potentially could eradicate the tumor. To obviate this serious limitation, we produced human EGFR-expressing transfectants of the F98 rat glioma (16), which is only weakly immunogenic (17) and has been incurable by all therapeutic modalities (18), except boron NCT (19), and most recently with a combination of cis-platinum and monochromatic synchrotron X-rays (20). Using F98 glioma cells transfected with the wild-type human EGFR gene (F98EGFR), we have evaluated boronated EGF (8, 12) and the mAb cetuximab, also known as IMC-C225, as delivery agents and established proof of principle that a significant therapeutic gain could be obtained following boron NCT (12, 13, 16).

Wild-type EGFR is overexpressed not only in a variety of human malignancies but also in normal liver and spleen, which can take up significant amounts of radiolabeled anti-EGFR mAb (21) or EGF (22, 23) following systemic administration. On the other hand, EGFRvIII, which is encoded by a gene with an in-frame deletion of exons 2 to 7, producing a mRNA species with an 801-bp deletion (24), is a more specific target for therapeutic agents (25, 26). This mutation produces a novel glycine residue at position 6 between amino acid residues 5 and 274 of wild-type EGFR, which results in constitutive, ligand-independent activation of the protein (27). EGFRvIII has been found in ~50% of human gliomas (28) but not in normal tissues, including those that express wild-type EGFR. Because EGFRvIII seems to be a truly tumor-specific target, several mAbs, including DH8.3 (28), 806 (29), and L8A4 (25, 26, 30), have been produced for diagnostic and therapeutic purposes.

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The goals of the present study were (a) to develop and characterize a syngeneic rat glioma model expressing EGFRvIII and (b) to study molecular targeting of this receptor by mAb L8A4, which is being evaluated by us as a boron delivery agent for NCT. In the present report, we describe the production and in vitro and in vivo characterization of a variant of the F98 glioma, designated F98npEGFRvIII, which expresses a nonconstitutively phosphorylated form of the receptor. This has been produced by transfecting wild-type F98 glioma cells (F98WT) with an expression vector containing a gene that encodes human EGFRvIII. Using this model, we have evaluated the in vivo biodistribution of mAb L8A4 as a potential tumor targeting agent following systemic, i.t., and convection enhanced delivery (CED). The data presented in this report suggest that potentially therapeutic amounts of boronated antibody can be delivered i.c. for NCT of this brain tumor.

MATERIALS AND METHODS

Expression Vectors. RNA was isolated from a tissue sample of human glioblastoma multiforme using RNeasy columns (Qiagen, Inc., Studio City, CA). EGFRvIII cDNA was synthesized by reverse transcription-PCR with SuperScript II (Life Technologies, Grand Island, NY). PCR reactions with Taq2000 DNA polymerase, Taq Extender, and PfuTurbo DNA polymerase (Stratagene, La Jolla, CA) were carried out using primers corresponding to the upstream start and downstream termination sites. PCR products were separated on 0.8% TAE agarose gels, and bands corresponding to wild-type EGFR and EGFRvIII forms were excised, sequenced, and introduced into the pLXIN vector. This contained a CA) containing the murine Moloney leukemia virus promoter to direct expression of the inserted DNA sequences. The vector also contains a neomycin insert (16). F98 cells were transfected with the pLXIN vector containing (1) wild-type EGFR, (2) a neomophosphorylated EGFRvIII mutant, designated npEGFRvIII, and (3) a wild-type EGFR insert, which was isolated and cloned into the pLXIN vector. This contained a glutamic acid-to-glycine substitution at amino acid position 423 and an A-to-G substitution at nucleotide position 1,268.5

Transfection of the F98 Glioma. The F98 rat glioma cell line was derived from CD-Fischer 344 rats treated with N-ethyl-N-nitrosourea and has been described by us in detail elsewhere (5). Non-adherent F98 cells were transfected with the pLXIN vector containing a neomycin insert (neo), wild-type EGFR, or npEGFRvIII cDNA using LipofectAMINE reagent (Life Technologies) according to the manufacturer’s instructions. Transfектants were treated with Geneticin (G418, Sigma-Aldrich, St. Louis, MO) at a concentration of 600 μg/mL and resistant clones were isolated with cloning discs (Bel-Art Products, Inc., Pequannock, NJ). Cell lines were propagated in 100-mm tissue culture plates in DMEM with 10% fetal bovine serum, 50 units/mL penicillin, and 50 μg/mL streptomycin at 37°C in 5% CO₂ with two to three medium changes per week. G418-resistant cell lines were screened by Western blot analysis. Cells were lysed in radioimmunoprecipitation assay buffer containing 50 mmol/L Tris-HCl (pH 7.5), 0.25% sodium deoxycholate, 1% NP40, 1 mmol/L sodium orthovanadate, 1 mmol/L sodium fluoride, 1 mmol/L EGTA, and 1 μg/mL each of pepstatin, leupeptin, and aprotinin. Lysates were sonicated for 30 seconds and centrifuged to remove debris, following which the samples, containing 50 μg total protein, were boiled and applied to 5% SDS-PAGE. Equal amounts of protein were transferred to Nytran membranes (Schleicher & Schuell Bioscience, Inc., Keene, NH) and were probed with anti-EGFRvIII antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Specific binding was detected with CDP-STAR chemiluminescent secondary substrate antibodies conjugated to alkaline phosphatase (Bio-Rad Laboratories, Inc., Hercules, CA).

Quantitative Fluorescence-Activated Cell Sorting Analysis of Receptor Expression of F98npEGFRvIII. The number of EGFR and npEGFRvIII sites expressed by the transfected F98 cells was determined by quantitative fluorescence-activated cell sorting (FACS) using Quantum Simply Cellular system (Flow Cytometry Standards Corp., San Juan, Puerto Rico). mAb L8A4, which is a murine mAb of IgG1 subclass that binds specifically to EGFRvIII but not to wild-type EGFR (26), was used to determine the number of npEGFRvIII sites. mAb EGFR-1 (BD PharMingen, San Jose, CA), which is an IgG2b mAb directed against the extracellular domain of wild-type EGFR and is unreactive with EGFRvIII, was used to determine the number of EGFRWT site density. Background binding was determined by inclusion of fluoresceinated, irrelevant IgG1 and IgG2b isotype controls. F98WT, F98EGFR, and F98npEGFRvIII cell lines were analyzed by quantitative FACS. One million cells were incubated for 30 minutes at 4°C with either 5 μg/mL mAb L8A4 or an isotype-matched negative control diluted in PBS containing 1% bovine serum albumin.

Murine mAb L8A4, directed against human EGFRvIII, was used to determine the number of receptor sites, and mAb EGFR-1 was used to determine the number of wild-type receptors. Direct fluorescenceimination of the mAbs was done by dialysis against 115 mmol/L sodium phosphate buffer (pH 7.4), with the protein concentration adjusted to 1 mg/mL, followed by the addition of 70 mg N-hydroxysuccinimide ester of FITC (Pierce, Rockford, IL) in 70 mL DMSO. After a 4-hour incubation at ambient temperature, the solution was dialyzed at 4°C against Dulbecco’s PBS. The fluoresceinated bioconjugates were stable at 4°C for 90 to 180 days. The microbead solution was a mixture of five uniform populations of the same size that had either no or serially increasing capacities to bind murine or human IgG. Incubation of the bead sample with an identical aliquot of fluoresceinated mAb, used for cell analysis, permitted the extrapolation of the number of antibody molecules bound per cell. Assuming a 1:1 stoichiometry for EGFR and EGFRvIII, the number of receptor sites was expressed as a population mean. The optimal concentrations of the fluoresceinated mAbs were determined by repeated titrations of mAb L8A4 versus NR6M cells, which expressed EGFRvIII, and mAb EGFR-1 versus NR6W cells, which expressed wild-type EGFR (27). The minimum concentrations that consistently saturated the available sites on 1 × 10⁶ cells were 5 μg/mL for mAb L8A4 and 10 μg/mL for EGFR-1. Background binding was determined by inclusion of fluoresceinated, irrelevant isotype controls (IgG1 and IgG2b, respectively) and negative cell controls (NR6W for L8A4 and NR6M for EGFR-1). Microbeads, calibrated for murine or human IgG binding, were labeled for 2 hours at 4°C with 300 μL fluoresceinated mAb (5-10 μg/mL), washed twice with ice-cold PBS containing 1% bovine serum albumin.

5 F.B. Furnari, unpublished data.
and resuspended in 0.5 mL of 0.5% paraformaldehyde for analysis on a FACSsort equipped with Lysys software (Becton Dickinson, San Jose, CA). Receptor density was analyzed by interpolation with microbead standard curves using QuickCal software. The estimated numbers of receptor sites obtained by quantitative flow cytometry were concordant with those obtained in both recent and past studies using iodinated antibodies or EGF in standard Scatchard analyses (26). In general, values <1 × 10⁴ were not considered positive, because this was only ~2 times greater than the assay detection threshold.

**L8A4 Receptor Binding Assay and Scatchard Analysis.** Radiolabeling of mAb L8A4 with ¹²⁵I was carried by the iodo-oxidation method using Iodo-Gen precoated idionation tubes according to the procedure described by the manufacturer (Pierce). F98WT (or parental) glioma cells were propagated in DMEM containing glucose, l-glutamine, and 10% fetal bovine serum (Life Technologies). F98npEGFRvIII cells were grown in the same medium supplemented with 200 µg/mL G418. The receptor binding activity of L8A4 with either F98WT or F98npEGFRvIII cells was quantified by a direct binding assay, briefly described as follows. F98 cells (~ 5 × 10⁵/well) were seeded into 24-well flat-bottomed plates (Corning, Inc., Corning, NY) and allowed to attach overnight, following which they were washed twice with serum-free DMEM and incubated for 2 hours in the same medium. DMEM then was removed and varying concentrations of ¹²⁵I-L8A4 ranging from 5 to 150 ng were added to triplicate wells and incubated at ambient temperature in an atmosphere containing 95% air and 5% CO₂ for 2 hours. The cells then were washed twice with PBS and harvested using 0.5 mmol/L EDTA in PBS (pH 7.2), and cell-associated radioactivity was determined by γ-scintillation counting using a well counter (model 1185, Tm Analytic, Elk Grove Village, IL).

**Tumorigenicity of the F98npEGFRvIII Glioma.** The tumorigenicity of the F98npEGFRvIII glioma transfectants was evaluated and compared with that of F98 parental cells in syngeneic CD-Fischer rats (Charles River Laboratories, Wilmington, MA). All animal studies were done in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, DC, 1996) and the protocol was approved by the Institutional Laboratory Care and Use Committee by the Ohio State University. Rats were stereotactically implanted with 10⁵, 10⁴, or 10³ cells F98npEGFRvIII or F98WT in 10 µL serum-free DMEM containing 1.2% to 1.4% agarose with a gelling temperature of <30°C. Cells were injected over 10 to 15 seconds through a central hole in a plastic screw (Arrow Machine Manufacturing, Inc., Richmond, VA) into the right caudate nucleus using a procedure described in detail elsewhere (10). Rats were observed daily and weighed thrice a week following tumor implantation to monitor their clinical status. As determined in previous studies with the F98 glioma (19), the combination of sustained weight loss, ataxia, and periportal 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 euthanization plus 1 day.

**Histologic and Immunohistochemical Characterization of the F98npEGFRvIII Glioma.** Rats bearing i.c. implants of F98WT or F98npEGFRvIII gliomas were euthanized at that point in time when they showed clinical signs of a progressively growing brain tumor. The brains were removed and fixed in 10% buffered formalin, following which they were embedded in paraffin. Sections (4 µm) were cut and stained with H&E for routine histologic examination and immunostained for EGFRvIII, wild-type EGFR, glial fibrillary acidic protein, and the proliferation marker Ki-67. Immunostaining for EGFRvIII and wild-type EGFR was carried out on either frozen or paraffin sections. For frozen sections, brains were embedded in Tissue Tek OCT compound (Sakura Finetek, Torrance, CA), snap frozen in the vapor phase of liquid nitrogen, and stored at −70°C until further processing. Sections (4 µm) were cut on a cryostat and fixed in ice-cold acetone for 10 minutes, following which they were air dried for an additional 10 minutes. Immunostaining of formalin-fixed, paraffin-embedded tissue with mAbs was done on 4-µm thick sections that were deparaffinized in sequential solutions of xylene and ethanol. Sections were blocked for endogenous peroxidase activity by incubation with a mixture of 0.3% H₂O₂ in absolute methanol for 10 minutes. They were rehydrated in PBS for 10 minutes and blocked by exposing them to 10% normal rabbit serum for 30 minutes at ambient temperature followed by exposure to either anti-EGFR mAb (Zymed, South San Francisco, CA) at a 1:1,000 dilution or L8A4 at a 1:200 dilution for 2 hours at ambient temperature. Slides were washed in PBS and the appropriate dilution of biotinylated rabbit anti-mouse IgG (DAKO Corp., Carpinteria, CA) was applied. The slides then were incubated for 30 minutes at ambient temperature, washed again in PBS, and exposed to horseradish peroxidase-avidin complex (ImmunoPure Ultra-Sensitive ABC Staining kit and Metal Enhanced DAB Substrate kit, Pierce) for 30 minutes, washed with PBS, and developed with diaminobenzidine.

**Magnetic Resonance Imaging of Brains of Glioma-Bearing Rats.** Rats bearing F98WT or F98npEGFRvIII gliomas were imaged using a Varian Unity Inova Magnetic Resonance System (Varian, Palo Alto, CA) beginning 8 days after implantation of 10⁵ glioma cells. Following implantation, the tumor volume was measured using T2- and T1-weighted magnetic resonance imaging (MRI) every second day (31). The T2-weighted images were acquired using multislice fast spin echo (FSE). The acquisition variables were recycle time/echo time (TR/TE) = 4,000/60 ms, in-plane field-of-view (FOV) = 30 × 30 mm, 13 (1 mm) slices, separated by 1.5 mm, and an echo train length of 8 for a total imaging time of 6 minutes. The T1-weighted images were acquired using multislice fast spin echo (FSE). The acquisition variables were recycle time/echo time (TR/TE) = 1,000/15 ms, in-plane FOV = 30 × 30 mm, 13 (1 mm) slices were separated by 1.5 mm with a total acquisition time of 8 minutes. All images were transferred to a PC running MATLAB for postprocessing and volume calculation. Images were displayed on an in-house graphical user interface allowing the tumor to be “circled” on each slice. The previously described software (31) was used to calculate the tumor volume from these regions of interest.

**Tissue blood flow (TBF) maps were calculated on a pixel-by-pixel basis using the following equation:**

\[\text{TBF} = \frac{\lambda(S_o - S_i)}{2T_1T_2} \]

where \(S_o\) is the signal from a perfusion-weighed FSE image, \(S_i\) is the signal from a control FSE image, \(\lambda\) is the blood/tissue partition function (0.9), \(\alpha\) is the efficiency of spin inversion (0.8), and \(T_1\) was the tissue.
spin-lattice relaxation time. Perfusion weighting was achieved using a continuous arterial spin labeling technique (32). Briefly, the magnetizations of the blood spins were inverted using multiple adiabatic radiofrequency inversion pulses. The effect of these was to reduce the MRI signal intensity by an amount that was proportional to the blood flow (mL/100 g/min) in the image plane. The FSE variables were TR/TE = 4,000/15 ms, FOV = 30 × 30 mm, slice thickness = 1 mm, and an echo train length of 16 for a total acquisition time of 12 minutes. All images and T1 maps were transferred to a PC workstation running MATLAB for calculation and display of TBF maps. The TBF to the tumor was displayed as a color overlay (mL/100 g/min) on the proton-weighed control FSE image. The mean TBF for each animal was calculated from regions of interest containing the whole tumor within the image slice. The mean TBF for each of the groups was quantitatively compared using a two-tailed t test assuming equal variance.

In vivo Biodistribution of 131I-Labeled L8A4 following i.v. Injection. Paired-label immunolocalization studies, as described by Wikstrand et al. (33), were done in F98npEGFRvIII glioma-bearing Fischer rats 2 weeks after i.c. implantation of 10^5 cells. mAb L8A4 and an isotype-matched murine IgG1 negative control were radiolabeled with either 131I or 125I, respectively, using the Iodo-Gen method. A mixture containing 5 μCi 131I-L8A4 (5 μg) and an equal amount of 125I-IgG1 was injected i.v. into the penile vein and animals were killed at 6, 24, 48, and 72 hours following injection. Tumor and normal tissues, including brain, blood, liver, spleen, kidneys, lungs, heart, skin, and muscle, were collected, weighed, and subjected to γ-scintillation counting using a well counter for determination of radioactivity. Data were corrected for overlap of 131I and 125I signals and for the decay of the radioisotopes. Values for the percentage of injected dose per gram (%ID/g) tissue were derived using injection dose standards. Tumor localization indices (33) were calculated from the following equation: 131I-L8A4 / 125I-IgG1 (tumor) / 131I-L8A4 / 125I-IgG1 (blood).

CED versus i.t. Injection of 131I-Labeled L8A4. Fourteen days after i.c. implantation of 10^5 F98npEGFRvIII glioma cells, rats were anesthetized with ketamine/xylazine and placed in a stereotactic head frame (David Kopf Instruments, Tujunga, CA). Using the same stereotactic coordinates as those that had been used to implant the tumor, an infusion cannula was inserted into the entry port of the plastic screw, which had been embedded into the calvarium, and this was advanced into the tumor. CED was carried out as described by us in detail elsewhere (34). Rats were divided into three groups of four animals each. Group 1 received 131I-L8A4 (5 μCi/5 μg/10 μL) by i.t. injection; group 2 received 131I-L8A4 given by CED using a Harvard syringe pump (Harvard Instruments, Cambridge, MA) adjusted to deliver 0.33 μL/min for 30 minutes; group 3, which was a control group, received 131I-IgG1 by i.t. injection. The animals were euthanized at 24 hours and tumor, normal brain, liver, kidney, and blood were collected. The biodistribution of 131I-L8A4 was determined by γ-scintillation counting.

RESULTS

Characterization of the F98npEGFRvIII Rat Glioma Cell Line. F98 glioma clones, which stably expressed the nonphosphorylated human EGFRvIII gene, were isolated. The mutant receptor protein was identified by Western blot analysis using a polyclonal antibody directed against the tyrosine kinase domain (Santa Cruz Biotechnology) of both wild-type EGFR and EGFRvIII. This detected a 170-kDa protein band corresponding to the expected molecular weight of EGFR and a 140-kDa band corresponding to EGFRvIII for...
The tumorigenicity of F98npEGFRvIII glioma cells was compared labeled L8A4 and the results are shown in Fig. 2. F98npEGFRvIII (i.e., in the range of $10^4$ per cell). Localization of EGFRvIII derived from the apparent binding site concentration ($K_a$ of L8A4 bound to F98EGFR cells).

Both mAbs bound to F98WT in amounts similar to background levels of control IgG1. The bead values for IgG1, IgG2b, and mAb EGFR-1, which was reactive with the wild-type receptor. Data on the expression of EGFRvIII by F98 npEGFRvIII compared with F98WT glioma cells are summarized in Table 1. Because in quantitative FACS mere visual inspection of the peaks is not accurate, regression analysis of the data was done and compared with data obtained with calibrated fluorescent beads. The F98 npEGFRvIII glioma expressed $1.2 \times 10^5$ receptor sites per cell compared with undetectable levels on F98WT cells (i.e., in the range of $10^4$ per cell).

Localization of EGFRvIII on the cell surface was confirmed and quantified by FACS using mAb L8A4. Quantitative FACS analysis revealed that F98npEGFRvIII cells were positive with mAb L8A4 but not with mAb L8A4. This was derived from the apparent binding site concentration ($B_{max}$) and the $K_a$ of L8A4 was 6.62 $\pm$ 0.8 x 10$^4$ mol/L$^{-1}$. In contrast, there was only background binding of radiolabeled L8A4 to cells expressing wild-type EGFR.

### In vivo Tumorigenicity of the F98npEGFRvIII Glioma.

The tumorigenicity of F98npEGFRvIII glioma cells was compared with that of the parental F98WT glioma by implanting logarithmically incremental numbers of cells i.e. into syngeneic Fischer rats. All rats died following implantation and the survival times decreased linearly with logarithmically increasing numbers of cells (data not shown). Following implantation of $10^5$, $10^4$, and $10^3$ tumor cells, the corresponding mean ± SD survival time of rats was 23 ± 2, 17 ± 2, and 13 ± 2 days for the F98npEGFRvIII glioma compared with 24 ± 2, 19 ± 3, and 15 ± 2 days for corresponding numbers of F98WT cells. In another study, in which $10^5$ cells were implanted into groups of 10 animals each, the mean survival time of F98npEGFRvIII glioma-bearing rats was 16 ± 3 days (range 11-24 days) and 19 ± 4 days (range 15-26 days) for F98WT glioma-bearing rats. Although the mean survival times of animals bearing F98npEGFRvIII gliomas were shorter than those of rats bearing equal numbers of F98WT cells, these were not statistically significant as determined by the Wilcoxon-Gehan rank sum test ($P = 0.1$).

### Histologic and Immunohistochemical Characterization of the F98npEGFRvIII Glioma.

The morphology of the F98npEGFRvIII glioma was indistinguishable on H&E-stained sections from that of the F98WT glioma as described previously by us (35). The tumor was composed of a mixed population of spindle shaped cells with fusiform nuclei, frequently forming a whorled pattern of growth, and a smaller subpopulation of polygonal cells with round to oval nuclei (Fig. 3A). Mitotic figures were moderately frequent. Scattered throughout the tumor were foci of necrosis in which the faint outlines of cells could be discerned. The necrotic foci were surrounded by several layers of pallisading tumor cells. Infiltrating islands of tumor cells were seen at varying distances in the adjacent white matter, and usually, they surrounded a central capillary (Fig. 3B).

Immunostaining of the F98npEGFRvIII glioma with mAbs directed against glial fibrillary acidic protein, Ki-67, and wild-type EGFR (Fig. 3D) all were negative. In contrast, immunostaining was positive with L8A4 (Fig. 3C), although this was patchy on paraffin sections but uniform on frozen sections of the tumor.

### Determination of In vivo Tumor Growth Rate and Blood Flow.

MRI studies were carried out in a small subset of animals to noninvasively quantify tumor growth rates as a function of time.

### Table 1 Expression of EGFR and npEGFRvIII as determined by FACS

<table>
<thead>
<tr>
<th>Cell line</th>
<th>EGFR wild-type*</th>
<th>npEGFRvIII†</th>
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<tbody>
<tr>
<td>F98WT</td>
<td>3.8 x 10⁵</td>
<td>2.6 x 10⁴</td>
</tr>
<tr>
<td>H898</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>F98npEGFRvIII</td>
<td>ND</td>
<td>ND</td>
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*Wild-type EGFR expression was determined using mAb EGFR-1, which is a murine IgG2b directed against the extracellular domain of wild-type EGFR and is nonreactive with npEGFRvIII.

†EGFRvIII expression was determined using mAb L8A4, which is a murine IgG1 that binds specifically to the mutant receptor but not wild-type EGFR.

H898, which was used as a control cell line, was (+) for both wild-type EGFR and npEGFRvIII.

Values in the range of $10^4$ receptor sites were considered nondetectable because this was only two times greater than the detection threshold of the assay.

### Fig. 2 Cellular binding of mAb L8A4 to F98npEGFRvIII (■) and F98EGFR (◇) glioma cells. Varying amounts of $^{125}$I-L8A4 (5-150 ng) were incubated with F98npEGFRvIII or F98EGFR cells at ambient temperature for 2 hours, following which cell-bound radioactivity was determined by γ-scintillation counting. Based on the amount of $^{125}$I-L8A4 bound, it was calculated that the F98npEGFRvIII glioma expressed $1.20 \pm 0.08 \times 10^5$ receptor sites per cell. This was derived from the apparent binding site concentration ($B_{max}$) and the $K_a$ of L8A4 with F98npEGFR-1, which was reactive with the wild-type receptor. Both mAbs bound to F98WT in amounts similar to background levels of control IgG1. The bead values for IgG1, IgG2b, and EGFR-1 were quite similar, which reflected a FITC/protein ratio that usually fell in the range of 3.8 to 4.6. L8A4 bound more FITC (FITC/protein ratio ~5.7-6.8), which accounted for the L8A4 profile being shifted to the right of the control IgG1 profile for the three cell lines. Nonphosphorylated EGFRvIII expression was determined using mAb L8A4, which is a murine IgG2b directed against the extracellular domain of type EGFR and npEGFRvIII.

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Immunostaining of the F98npEGFRvIII glioma with mAbs directed against glial fibrillary acidic protein, Ki-67, and wild-type EGFR (Fig. 3D) all were negative. In contrast, immunostaining was positive with L8A4 (Fig. 3C), although this was patchy on paraffin sections but uniform on frozen sections of the tumor.

**In vivo Tumorigenicity of the F98npEGFRvIII Glioma.** The tumorigenicity of F98npEGFRvIII glioma cells was compared with that of the parental F98WT glioma by implanting logarithmically incremental numbers of cells i.e. into syngeneic Fischer rats. All rats died following implantation and the survival times decreased linearly with logarithmically increasing numbers of cells (data not shown). Following implantation of $10^5$, $10^4$, and $10^3$ tumor cells, the corresponding mean ± SD survival time of rats was 23 ± 2, 17 ± 2, and 13 ± 2 days for the F98npEGFRvIII glioma compared with 24 ± 2, 19 ± 3, and 15 ± 2 days for corresponding numbers of F98WT cells. In another study, in which $10^5$ cells were implanted into groups of 10 animals each, the mean survival time of F98npEGFRvIII glioma-bearing rats was 16 ± 3 days (range 11-24 days) and 19 ± 4 days (range 15-26 days) for F98WT glioma-bearing rats. Although the mean survival times of animals bearing F98npEGFRvIII gliomas were shorter than those of rats bearing equal numbers of F98WT cells, these were not statistically significant as determined by the Wilcoxon-Gehan rank sum test ($P = 0.1$).
As shown in Fig. 4A to D, this consisted of T2-weighted multislice imaging along with T1 post-gadolinium contrast scans. Based on MRI measurements, the mean ± SE tumor volumetric doubling times for the F98WT and F98npEGFRvIII gliomas were 59.8 ± 4.8 and 52.0 ± 3.3 hours (n = 4), respectively, which were not significantly different (P > 0.05). Plots of mean tumor volume versus time for the F98WT and F98npEGFRvIII gliomas are shown in Fig. 4E and F. The slopes of these plots were 0.11 ± 0.01 and 0.13 ± 0.01 for the F98WT and F98npEGFRvIII gliomas, respectively, and were statistically equivalent (P > 0.25). Quantitative perfusion TBF maps were calculated for each animal (n = 4 per group) and representative maps for the F98WT and F98npEGFRvIII gliomas are shown in Fig. 3E and F. Mean perfusion values for the F98WT and F98npEGFRvIII glioma groups were 50 ± 8 and 36 ± 7 mL/100 g/min (Fig. 4E and 4F) respectively, and these were not statistically different from one another (P > 0.05).

Biodistribution of Systemically Administered L8A4 in F98npEGFRvIII Glioma-Bearing Rats. Following i.v. administration of 131I-L8A4 and 125I-IgG1 to F98npEGFRvIII glioma-bearing rats, localization of the antibody was determined by paired-label analysis and the data are summarized in Figs. 5 and 6. As determined by the Wilcoxon log rank sum test, the tumor uptake values of 131I-L8A4 and 125I-IgG1 were significantly different from each other (P < 0.01). For 131I-L8A4, the % ID/g tumor at 6, 24, 48, and 72 hours ranged from a peak of 4.2% ID/g at 48 hours to a low of 4.2% ID/g at 6 hours compared with that of 125I-IgG1, which ranged from 0.9% to 2.2% (Fig. 5). The biodistribution profiles of 131I-L8A4 in rats bearing F98npEGFRvIII are shown in Fig. 6A. The tumor uptake of L8A4 was much higher than that of any other tissue beginning at 24 hours following i.v. injection. Using a nonlinear regression program, pharmacokinetic analysis was done on geometric mean blood concentrations calculated from data shown in Fig. 6A. Between 6 and 72 hours after i.v. administration of either 131I-L8A4 or 125I-IgG1, there was a monoexponential decline in radioactivity with a t1/2 of 25 hours. Tumor/brain ratios ranged from 9.9 to 25 for the ipsilateral tumor-bearing cerebral hemisphere and 17 to 66 for the contralateral hemisphere (Fig. 6B). Tumor/blood ratios were 9.7 and 8.5 at 48 and 72 hours, respectively (Fig. 6B). Specific localization in the tumor, relative to blood levels of L8A4 and to the nonspecific localization of IgG1, was determined by a localization index. These ranged from 1.3 at 6 hours, 3.5 after 24 hours, peaked at 48 hours, with a localization index of 6.6 indicating a high degree of specificity, and declined to 4.2 at 72 hours.

Localization of L8A4 following i.t. Injection and CED. The radiolocalization profiles of 131I-L8A4 and 131I-IgG1 in F98npEGFRvIII glioma-bearing rats at 24 hours after i.t. injection or CED are summarized in Table 2. As determined by γ-scintillation counting at 24 hours following CED, 59.8% ID/g of L8A4 was localized in F98npEGFRvIII gliomas compared with 39.7% and 21.4% ID/g for the control IgG1 after direct i.t. injection. CED was significantly superior to i.t. injection as a means to increase the uptake of 131I-labeled L8A4 (P < 0.01, Wilcoxon log rank sum test) within the tumor, although the tumor/blood ratios were equivalent (6.4 versus 6.8). In contrast, the tumor uptake of 131I-L8A4 24 hours following CED was seven times greater than that following i.v. injection (59.8% versus 7.7% ID/g; as shown in Fig. 6.
DISCUSSION

In the present study, we have produced a highly tumorigenic variant of the F98 glioma, designated F98npEGFRvIII, which expressed nonphosphorylated EGFRvIII protein. Following i.c. implantation of as few as 1,000 cells, this tumor had 100% lethality in Fischer rats. As determined by both quantitative FACS analysis and a radioligand binding assay using 125I-labeled mAb L8A4, F98npEGFRvIII cells expressed 1.20 \times 10^5 receptors per cell and the \( K_d \) of radiolabeled antibody was 6.62 \times 10^5 mol/L^{-1}. The in vivo morphology of the F98npEGFRvIII glioma was indistinguishable from that of the parental F98WT glioma, which has been described previously in detail by us (35). However, F98npEGFRvIII cells showed strong membrane immunostaining with mAb L8A4, whereas F98WT cells were negative. The tumorigenicity of the F98npEGFRvIII glioma was similar to that of F98WT glioma as evidenced by both equivalent mean survival times of animals that received logarithmically incremental numbers of tumor cells and sequential MRI measurements of tumor growth volume, which indicated that they had virtually identical in vivo doubling times. Furthermore, magnetic resonance perfusion studies revealed that these tumors had similar blood flow values.

Whether expression of human EGFRvIII by the F98 rat glioma could evoke a protective immune response has been addressed in a separate study (36). It was shown that repeated vaccination of Fischer rats with a multiple antigenic peptide of a tumor-specific sequence of amino acids of human EGFRvIII not evoke a tumor protective immune response.

It has been reported previously that EGFRvIII expression in vivo enhanced tumor growth (37–39). The similarity in tumorigenicity and growth rates between F98WT and F98npEGFRvIII gliomas is consistent with the markedly reduced phosphorylation of npEGFRvIII. Studies by Cavenee’s group have shown that human U87MG glioma cells transfected with the EGFRvIII gene expressed 1 \times 10^6 to 3 \times 10^6 receptor sites per cell (37), which was 10 times higher than that we have observed with the F98npEGFRvIII cell line. U87ΔEGFR transfecteds had enhanced in vivo tumor growth following s.c. or i.c. implantation into nude mice (37, 40), whereas the in vitro growth of the parental and ΔEGFR transduced cells was similar. It was suggested that the mutant gene might have altered in vivo interactions between tumor cells and their microenvironment (37). Overexpression of EGFRvIII results in EGF-independent constitutive phosphorylation of tyrosine residues of the receptor (27, 37), which at least in part is mediated through the Ras-Shc-Grb2 pathway (38). Experiments in which U87MG parental and EGFRvIII glioma cells were admixed in varying ratios and implanted s.c. or i.c. resulted in an overgrowth of EGFRvIII-expressing cells (41).

### Table 2  Radiolocalization of 131I-labeled mAb L8A4 in rats bearing F98npEGFRvIII gliomas at 24 hours following either i.t. injection or CED

<table>
<thead>
<tr>
<th>mAb/route</th>
<th>Tumor</th>
<th>% ID/g*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Brain (ipsilateral)</td>
<td>Brain (contralateral)</td>
</tr>
<tr>
<td>L8A4/i.t.</td>
<td>39.5 ± 8.4</td>
<td>6.8 ± 1.2</td>
</tr>
<tr>
<td>L8A4/CED</td>
<td>59.8 ± 7.2</td>
<td>13.2 ± 7.1</td>
</tr>
<tr>
<td>IgG1/i.t.</td>
<td>21.4 ± 5.1</td>
<td>5.6 ± 5.3</td>
</tr>
</tbody>
</table>

*Animals were euthanized at 24 hours following administration and radioactivity was determined by γ-scintillation counting.
†Lateral designates the normal brain of the tumor-bearing right cerebral hemisphere and contralateral designates the non-tumor-bearing left cerebral hemisphere.
‡Animals received 131I-L8A4, given by CED using a Harvard syringe pump, adjusted to deliver 0.33 Ci/min for 30 minutes.
§Animals received 5 μg (5 μCi) of either 131I-L8A4 or 125I-IgG1 in a volume of 10 μL injected i.t., given over 2 minutes via the entry port of a plastic screw that had been embedded into the calvarium at the time of tumor implantation.

### Fig. 4  In vivo magnetic resonance images of rats bearing F98 gliomas.

A and B, F98WT glioma images corresponding to T1-weighed/post gadolinium contrast and T2-weighed contrast, respectively. C and D, images from a F98npEGFRvIII glioma corresponding to T1-weighed/post gadolinium contrast and T2-weighed contrast, respectively. E and F, mean tumor volume plots (animals per point) versus time for the F98WT glioma and the F98npEGFRvIII glioma, respectively.
Because the F98 npEGFRvIII glioma did not have enhanced tumorigenicity or an *in vivo* growth rate compared with the F98WT tumor, this could be very useful for studies in which F98WT and F98npEGFRvIII cells were admixed. This could be advantageous for studies focusing on *in vivo* tumor targeting by mAbs, where it would be desirable to have a relatively constant ratio of EGFR wild-type and EGFRvIII-expressing cells rather than a constantly changing one, as would occur if EGFRvIII was constitutively phosphorylated. Both we and Furnari and Cavenee (unpublished data) also have produced F98EGFRvIII transfectants that show constitutive autophosphorylation of EGFRvIII, thereby providing a unique pair of EGFRvIII-expressing glioma cell lines, one expressing and the other lacking constitutive autophosphorylation.

The second goal of this study was to determine the *in vivo* tumor localizing properties of L8A4 following administration to rats bearing i.c. implants of F98npEGFRvIII. Following i.v. administration, 
\[ 131^\text{I}-\text{L8A4} \] and 
\[ 125^\text{I}-\text{IgG1} \] glioma-bearing rats were injected i.v. with 5 \( \mu \text{Ci} \) 
\[ 131^\text{I}-\text{L8A4} \] and 5 \( \mu \text{Ci} \) 
\[ 125^\text{I}-\text{IgG1} \] and killed 6, 24, 48, and 72 hours. Columns, mean of four to five rats; bars, SD. For L8A4, the %ID/g tumor at 6, 24, 48, and 72 hours varied from a peak of 13.7 at 48 hours to a low of 4.2 at 6 hours compared with that of 
\[ 125^\text{I}-\text{IgG1} \] that ranged from 0.9 to 2.2.

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\[ 131^\text{I}-\text{L8A4} \] in the tumor peaked at 13.7% ID/g at 48 hours compared with 2.2% ID/g for 
\[ 125^\text{I}-\text{IgG1} \] and the localization index peaked at 6.6 at 48 hours. Because our MRI studies revealed that these tumors had similar blood flow values and permeability, based on gadolinium enhancement, this effectively ruled these out as possible explanations for the differences in uptake. Thus, the difference in retention of radiolabeled mAb L8A4 was due to its specific binding to F98npEGFRvIII glioma cells. Previous studies with L8A4 have shown that this mAb could specifically target s.c. implants of the human U87MGΔEGFR glioma in nude mice (42, 43). We now have shown specific targeting of i.c. implants of the F98npEGFRvIII glioma, and to the best of our knowledge, this is the first syngeneic EGFRvIII-expressing glioma model. As such, it could have great utility for studies on molecular targeting of this receptor by mAbs linked to tumoricidal agents, such as radionuclides (42, 43), toxins, or, as in the case of our own special interest (12), 
\[ ^{10}\text{B} \] for boron NCT (13). However, because there was no constitutive autophosphorylation of the receptor, this model would not be useful for studies with EGFRvIII targeting agents, such as small molecular weight receptor tyrosine kinase inhibitors, the mode of action of which is dependent on a biologically functional receptor (44).

We would like to make some comment concerning the delivery of L8A4 by either direct i.t. injection or CED. One major limitation of using anti-EGFR mAbs to treat patients with brain tumors has been that following systemic administration (45–47) exceedingly small quantities of the antibody localized in the tumor. This has been attributed to a combination of rapid clearance by the reticuloendothelial system and the blood-brain...
barrier, which prevented transendothelial passage of hydrophilic agents with a molecular weight in excess of ~250 Da, unless there is an active transport system (48). Direct i.c. delivery would minimize toxicity associated with systemic administration, bypass the blood-brain barrier, and reduce by orders of magnitude the amount of the antibody that would have to be given by systemic injection. Previously, we have shown that the amount of boronated EGF that localized in the tumor following i.v. injection was 0.06% at 24 hours and that this was insufficient to deliver the requisite amount of $^{10}$B to the tumor for boron NCT (10, 23). Our recent studies indicate that the tumor boron uptake can be further improved by CED (34). This is an innovative method to increase the uptake and distribution of both low and high molecular weight agents to the central nervous system by applying a pressure gradient to establish bulk flow during interstitial infusion (49–52). CED seems to have promise for improving brain tumor uptake of both high molecular weight agents, such as in the present study, or low molecular weight drugs, as have been reported recently by Degen et al. (53). They observed survival times >120 days for 9L gliosarcoma-bearing rats that received either carboptatin or gemcitabine by CED compared with survival times of 20 to 30 days following i.p. administration of the drugs. Similarly, it has recently been reported by Grossi et al. (54) that i.c. microinfusion (i.e., CED) of the mAb trastuzumab (Herceptin) increased the median survival time by 96% of nude mice bearing i.c. implants of a human HER-2-expressing breast cancer cell line compared with animals that received a control mAb. In a previous study, we found that the tumor uptake of $^{125}$I-labeled boronated EGF was increased 1.4 times following administration by CED compared with that obtained by i.t. injection (11) and was orders of magnitude greater than that obtained following systemic administration (23). For this reason, we have employed either direct i.t. injection or CED to administer mAb L8A4. The amount of $^{125}$I-L8A4 localized in the tumor following CED was increased 1.5 times over that obtained following i.t. injection. However, it remains to be determined if this also will improve the microdistribution within the tumor and ultimately the efficacy of EGFvIII targeting bioconjugates. We have previously carried out studies on the cellular uptake and microlocalization of boronated EGF (8) using electron spectroscopic imaging and electron energy loss spectroscopy (55), and either this technique or secondary ion mass spectrometry (56) will be used to determine the microdistribution of boronated bioconjugates (57) in future studies.

In conclusion, in the present study, we have described the production and characterization of an EGFvIII-expressing rat glioma that can be used for studies on molecular targeting of this receptor. This could be either with a mAb such as L8A4, which specifically recognizes EGFvIII, or with a mAb such as cetuximab (IMC-C225), which recognizes both wild-type EGF and EGFvIII (2). Because gliomas contain a heterogeneous population of tumor cells, including those that may express either wild-type EGF or EGFvIII, this could be accomplished using a combination of agents, such as boronated EGF and L8A4, or boronated cetuximab, which would target both subpopulations (57). Studies investigating this approach are in progress.

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REFERENCES


40. Caveneck WK. Receptor driven paracrine interactions: tumor heterogeneity. 15th International Conference on Brain Tumor Research and Therapy; Sorrento, Italy; May 24–27, 2003.


Development of a Syngeneic Rat Brain Tumor Model Expressing EGFRvIII and Its Use for Molecular Targeting Studies with Monoclonal Antibody L8A4

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