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# EPIDERMAL GROWTH FACTOR (EGF) AS A POTENTIAL TARGETING AGENT FOR DELIVERY OF BORON TO MALIGNANT GLIOMAS

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## 1. INTRODUCTION

The majority of high grade gliomas express an amplified epidermal growth factor receptor (EGFR) gene, and this often is associated with an increase in cell surface receptor expression[1,2]. The rapid internalization and degradation of EGF-EGFR complexes, as well as their high affinity make EGF a potential targeting agent for delivery of $^{10}B$ to tumor cells with an amplified number of EGFR. Human glioma cells can expresses as many as $10^5$-$10^6$ EGF receptors per cell, and if these could be saturated with boronated EGF, then $>10^8$ boron atoms would be delivered per cell. Since EGF has a comparatively low molecular weight (~ 6 kD), this has allowed us to construct relatively small bioconjugates containing ~ 900 boron atoms per EGF molecule[3], which also had high affinity for EGFR on tumor cells. In the present study, the feasibility of using EGF receptors as a potential target for therapy of gliomas was investigated by *in vivo* scintigraphic studies using $^{131}I$- or $^{99m}Tc$-labeled EGF in a rat brain tumor model. Our results indicate that intratumorally delivered boron- EGF conjugates might be useful for targeting EGFR on glioma cells if the boron containing moiety of the conjugates persisted intracellularly. Further studies are required, however, to determine if this approach can be used for BNCT of the rat glioma.

## 2. MATERIALS AND METHODS

### 2.1 Cells

Rat glioma $C6_{EGFR}$ cells were produced by transfecting the C6 rat glioma with the gene encoding for EGFR, and as determined by a radioligand binding assay and Scatchard analysis, expressed 3 x $10^6$ receptors per cell[4].

### 2.2 Preparation of boronated EGF conjugates

"Starburst" dendrimers, which are composed of repetitive polyamido amino groups arranged in a starburst pattern, were boronated with $Na(CH_3)_3NB_{10}H_8NCO$ as previously described[5]. EGF was first modified with m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester (sMBS) and then linked to boronated starburst dendrimers, derivatized with N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP). The reaction mixture was passed through a Sephadex G-50 column and eluted with 0.1 M TRIS, 0.2 M NaCl buffer (pH=8.5). One ml fractions were collected and protein concentrations were determined spectrophotometrically by measuring absorbance at 280 nm and boron was quantified by means of direct current plasma atomic emission spectroscopy (DCP-

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AES). Fractions containing peak concentrations of both protein and boron were pooled and used in the studies described in the following sections. The final concentration of EGF was precisely determined using Water's pico-TAG system[6,7] for amino acid analysis of the boronated EGF (B-EGF).

### 2.3 Receptor binding assay

Reactivity of boronated and native EGF with $C6_{EGFR}$ cells was studied by means of a competitive binding assay. $C6_{EGFR}$ cells (~ 5 x $10^6$ cells/well) were seeded into 24 well plates (Corning, Corning, NY) and incubated with medium containing 0.185 nM of $^{125}$I-EGF and varying concentrations of native (0 - 83 nM) or boronated (0 - 410 nM) EGF at 4 °C for 90 min. The cells then were washed three times with cold phosphate buffered saline (PBS, pH=7.2) and cell associated activity was determined by gamma scintillation counting. Affinity constants ($K_A$) were calculated using a four-parameter logistic equation for fitting the competition curve to the experimental displacement data[8].

### 2.4 Radiolabeling of EGF

EGF was radioiodinated by means of the chloramine-T method. EGF was labeled with $^{99m}$Tc by first treating it with 2-iminothiolane (50 x molar excess) in order to introduce a thiol group into the aminoterminal and then reacting it, in reducing buffer, with 10 mCi of $^{99m}$Tc sodium pertechnate (Mallinckrodt Medical Inc., St. Louis, MO)[9].

### 2.5 *In vivo* scintigraphic studies.

$C6_{EGFR}$ cells ($10^5$) were implanted stereotactically into the right caudate nuclei of Fischer rats as previously described[4]. The distribution of radioactivity following intracerebral (i.c.) or intratumoral injection of $^{131}$I- or $^{99m}$Tc-EGF in normal or tumor bearing animals was determined by means of external scintigraphy using a Technicare 438 gamma camera.

## 3. RESULTS AND DISCUSSION

Boronated "starburst" dendrimers were conjugated to EGF using sMBS and SPDP as chemical linkers. Based on protein and boron determinations, it was calculated that the bioconjugates contained ~ 900 atoms of boron per EGF molecule. Assuming that $10^5$ - $10^6$ EGFR on each tumor cell could be saturated with boronated EGF, then > $10^8$ boron atoms would be delivered to tumor cell. As determined by competitive binding assays, 83 nM of native EGF produced a 98% decrease in $^{125}$I-EGF binding and 410 nM of boronated EGF produced a 96% decrease in $^{125}$I-EGF binding (Fig. 1). The calculated $K_A$ for native EGF was 9.1 ($\pm$3.3) x $10^8$ $M^{-1}$, compared to 8.55( $\pm$0.9) x $10^7$ $M^{-1}$ for B-EGF, indicating that linkage of the boronated "starburst" dendrimer to EGF produced a ~ 10-fold decrease in the $K_A$ of the conjugate. Nevertheless, the $K_A$ of the B-EGF compared favorably to the $K_A$ of MoAbs that have been used for *in vivo* targeting of radionuclides and toxins[10].

External scintigraphy, following injection of $^{131}$I-EGF, showed that $^{131}$I radioactivity disappeared more rapidly from the brain region of tumor bearing animals compared to non-tumor bearing ones (Fig. 2). This may have been due to dehalogenation of EGF following its internalization and

degradation by tumor cells. In contrast, following injection of $^{99m}$Tc-EGF, $^{99m}$Tc radioactivity persisted for longer periods of time in the brain region of $C6_{EGFR}$ bearing rats compared to non tumor bearing animals (Fig. 3).

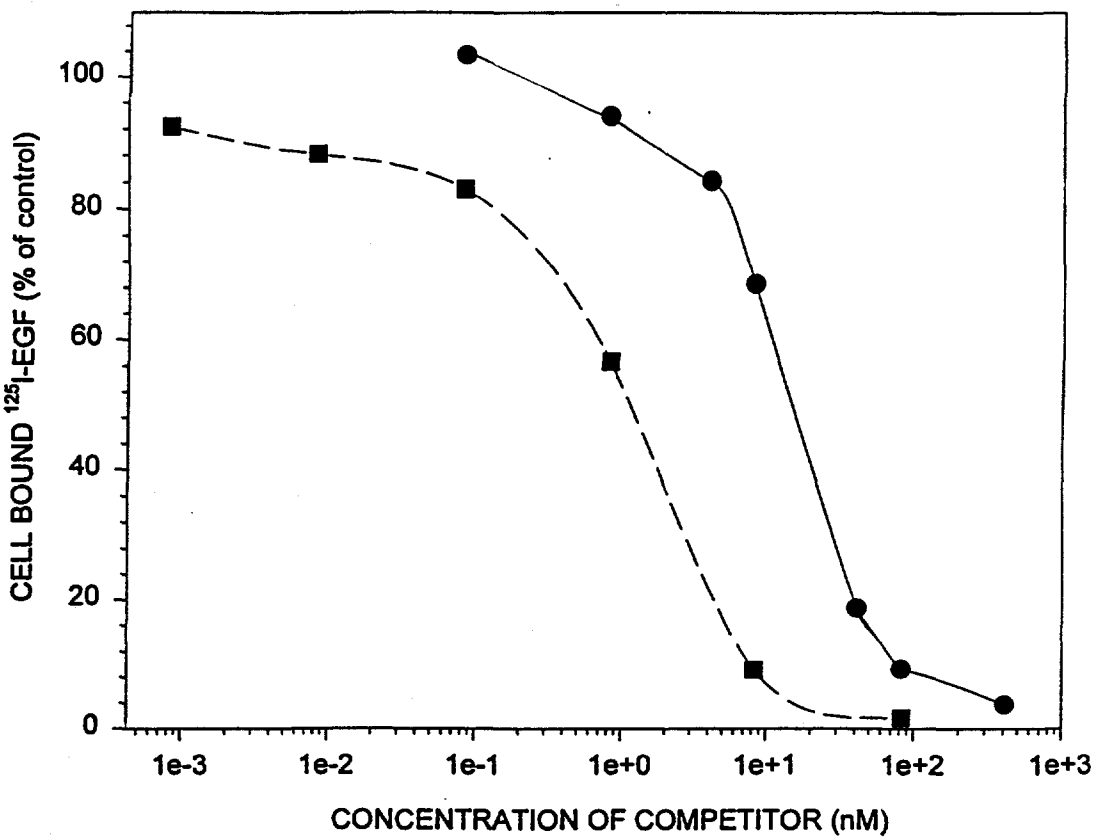


Figure 1. Inhibition of $^{125}$I-EGF binding obtained when increasing concentrations of native (■) or boronated (●) EGF were used as competitors. 5 x $10^5$ cells/well were incubated at 4° C for 90 min in medium containing 0.185 nM of $^{125}$I-EGF and various concentrations of native (0 - 83 nM) or boronated (0 - 410 nM) EGF. Then, the cells were washed in cold PBS and the cell associated activity was determined.

This suggested that either $^{99m}$Tc-EGF was not degraded or that $^{99m}$Tc radioactivity remained in tumor cells after degradation of $^{99m}$Tc-EGF. Intratumorally injected EGF most likely had been catabolized by the tumor cells, as suggested by faster disappearance of $^{131}$I from the brain, lower levels of radioactivity in the liver and more rapid accumulation of iodine in the thyroid. Following the injection of $^{99m}$Tc labeled EGF, ~ 60% of $^{99m}$Tc remained in the region of brain tumor for up to 12 hours, while in non-tumor bearing animals only ~ 20% of the injected radioactivity was detected in the brain region at six hours (Fig. 4). This is in agreement with results of our *in vitro* experiments with $^{131}$I- or $^{99m}$Tc-EGF, in which $^{131}$I radioactivity was rapidly released from the cells, while ~ 60% of $^{99m}$Tc radioactivity remained in the cells up to six hours (data not shown). Since intratumorally injected EGF may be catabolized by the tumor cells, it is essential that the boron containing molecules be designed so that they remain within the cells even after EGF has been degraded.

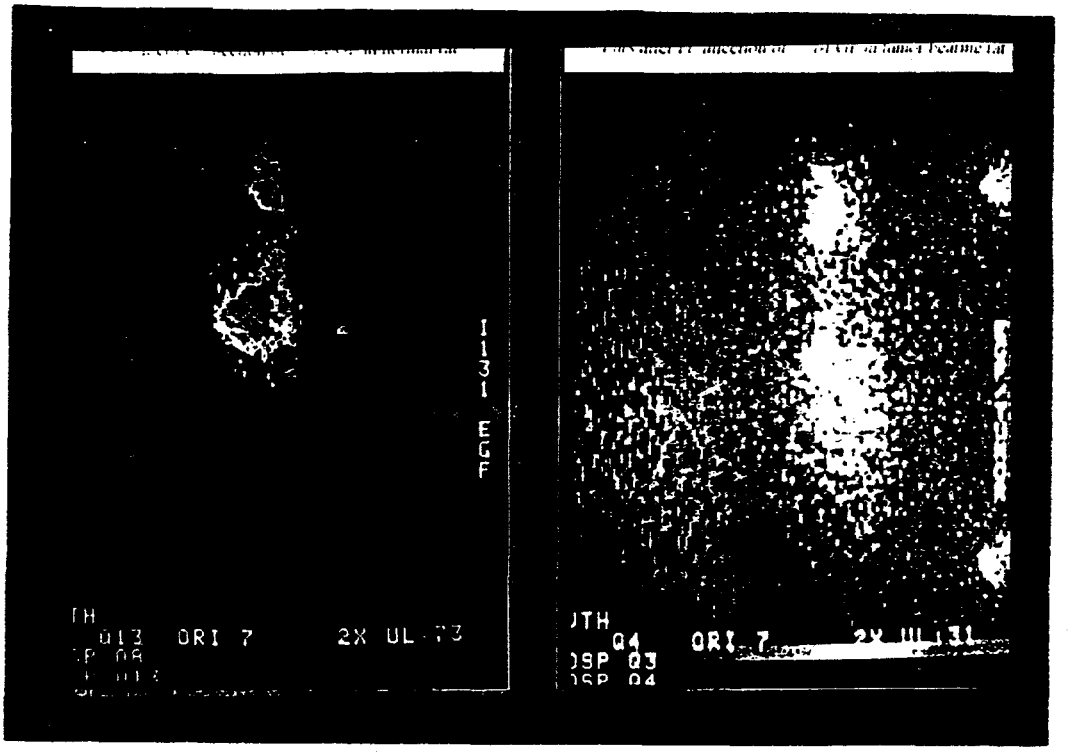


Figure 2. Scans of $^{131}I$ distribution 24 hrs after i.c. injection of ~ 5 $\mu$g of $^{131}I$-EGF (200 $\mu$Ci) in normal rats (left panel) or intratumoral injection of ~ 1 $\mu$g of $^{131}I$-EGF (60 $\mu$Ci) in tumor bearing rats (right panel).

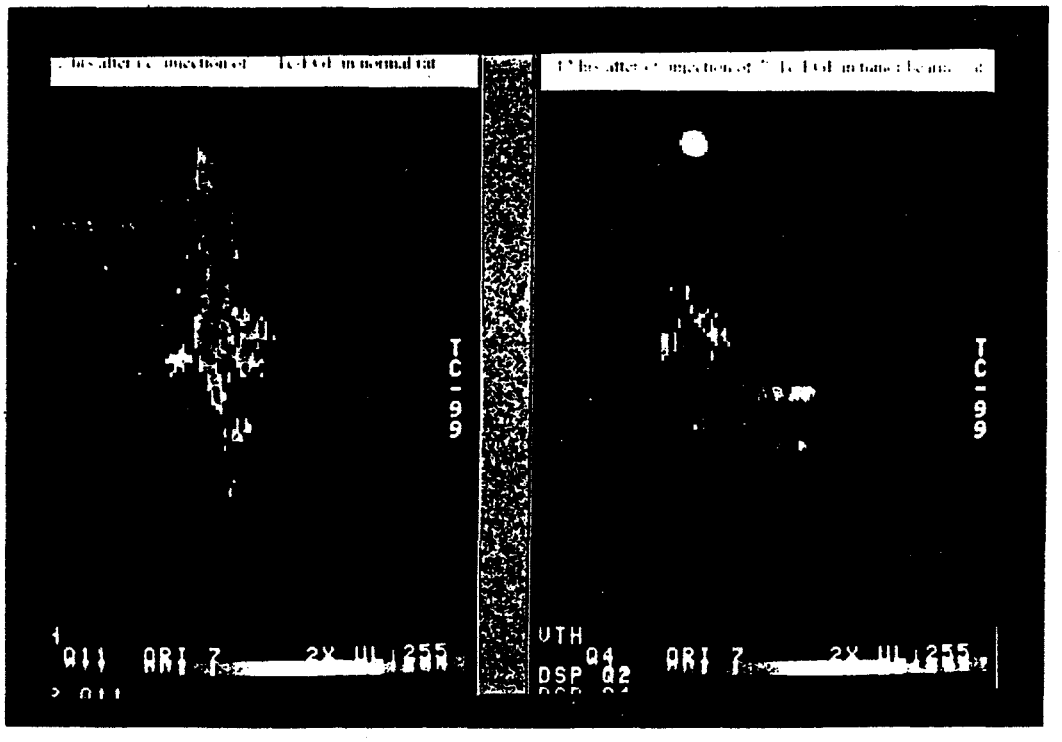


Figure 3. Scans of $^{99m}Tc$ distribution 12 hrs after i.c. injection of ~ 2.3 $\mu$g of $^{99m}Tc$-EGF (43 $\mu$Ci) in normal rats (left panel) or intratumoral injection of ~ 1.7 $\mu$g of $^{99m}Tc$-EGF (34 $\mu$Ci) in tumor bearing rats (right panel).

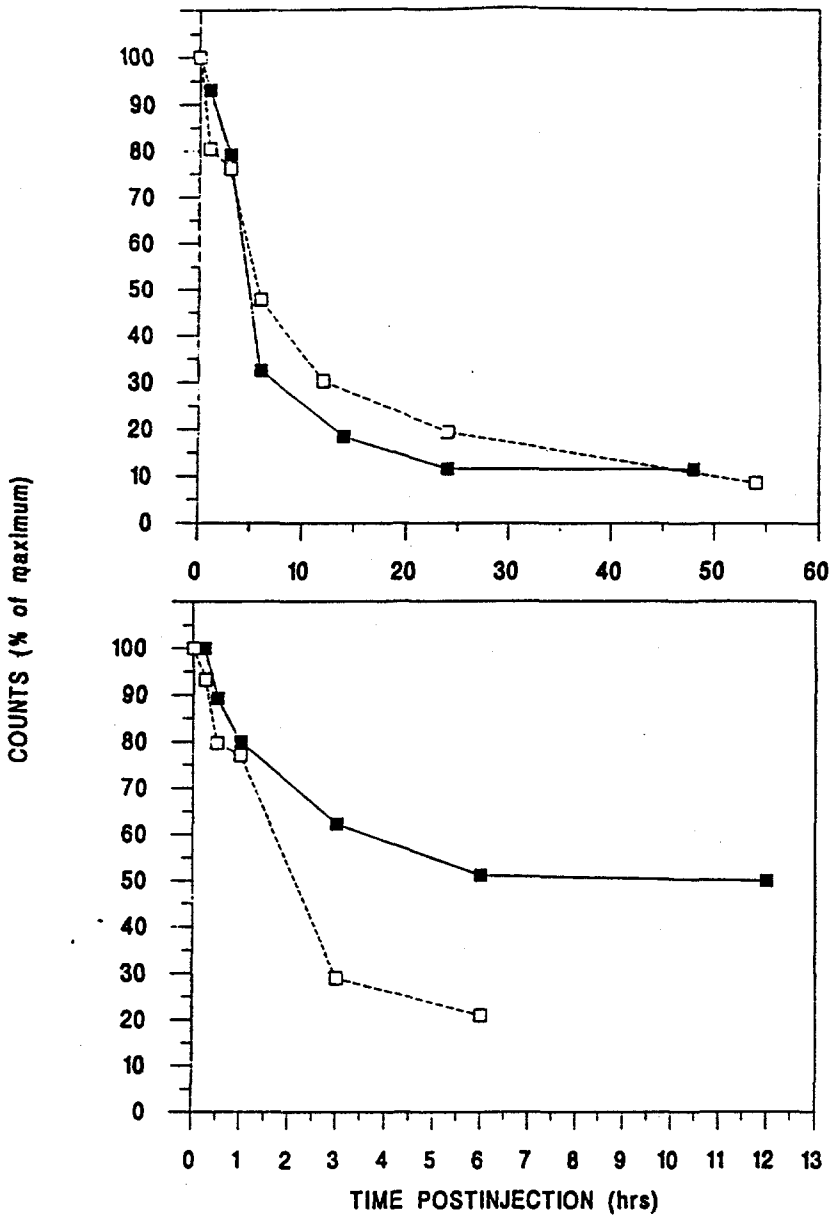


Figure 4. Quantitative evaluation of $^{131}I$ (upper panel) or $^{99m}Tc$ (lower panel) radioactivity retained in the brain region of normal (□) or tumor bearing (■) rats.

In order for boronated EGF to be useful as a targeting agent for BNCT of gliomas, the following criteria must be met: 1. the conjugates must retain their high affinity for EGF receptors *in vivo*; 2. following either systemic or local administration, sufficient amounts must reach glioma cells dispersed within normal brain; 3. the boron must persist intracellularly within glioma cells for a longer time than that required to clear from normal brain.

Since there may be considerable variability in the cellular expression of EGFR within any given glioma, combinations of low and high molecular weight delivery systems could be used to target different subpopulations of tumor cells. In order to determine the potential usefulness of EGF as a targeting agent for BNCT of gliomas, further *in vivo* studies will be carried out to define the biodistribution and pharmacokinetics of B-EGF following different routes of administration.

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