Growth Suppression of Intracranial Xenografted Glioblastomas Overexpressing Mutant Epidermal Growth Factor Receptors by Systemic Administration of Monoclonal Antibody (mAb) 806, a Novel Monoclonal Antibody Directed to the Receptor

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Abstract

A mutant epidermal growth factor receptor (variously called ΔEGFR, Δε2–7 EGFR, or EGFRvIII) containing a deletion of 267 amino acids of the extracellular domain is frequently highly expressed in human malignant gliomas and has been reported for cancers of the lung, breast, and prostate. We tested the efficacy of a novel monoclonal anti-ΔEGFR antibody, mAb 806, on the growth of intracranial xenografted gliomas in nude mice. Systemic treatment with mAb 806 significantly reduced the volume of tumors and increased the survival of mice bearing xenografts of U87 MG:ΔEGFR, LN-Z308:ΔEGFR, or A1207:ΔEGFR gliomas, each of which expresses high levels of ΔEGFR. In contrast, mAb 806 treatment was ineffective with mice bearing the parental U87 MG tumors, which expressed low levels of endogenous wild-type EGFR, or U87 MG.DK tumors, which expressed high levels of kinase-deficient ΔEGFR. A slight increase of survival of mice xenografted with a wild-type EGFR-overexpressing U87 MG glioma (U87 MG.wtEGFR) was effected by mAb 806 concordant with its weak cross-reactivity with such cells. Treatment of U87 MG.ΔEGFR tumors in mice with mAb 806 caused decreases in both tumor growth and angiogenesis, as well as increased apoptosis. Mechanistically, in vivo mAb 806 treatment resulted in reduced phosphorylation of the constitutively active ΔEGFR and caused down-regulated expression of the apoptotic protector, Bcl-XL. These data provide preclinical evidence that mAb 806 treatment may be a useful biotherapeutic agent for those aggressive gliomas that express ΔEGFR.

Introduction

mAbs3 with specificity for tumor-associated antigens have long been considered promising agents for the treatment of cancer (1–3). Such mAbs may have intrinsic antitumor activity, they may be used in combination with other therapies, or they can be used to deliver conjugated cytotoxic agents (2). Receptors for certain growth factors are potential targets for this approach because their location on the cell surface may make them accessible to antibodies the subsequent binding of which may block the growth-regulatory biological functions of the receptor (3–5). One notable example is the EGFR family member, HER-2/Neu (ErbB2), a transmembrane tyrosine kinase receptor frequently overexpressed in breast cancers (6, 7). The anti-HER-2 antibody, Herceptin, causes growth inhibition of HER-2-expressing cells in vitro and in vivo (8) and shows antitumor effects in clinical trials (7, 9, 10). The wtEGFR gene itself is also overexpressed in a wide variety of human epithelial tumors, including those of the breast, ovary, lung, and head and neck, as well as glioblastomas (4, 11, 12). Several anti-EGFR mAbs have been tested for radioimmunodiagnostic and radioimmunotherapeutic purposes, and the inhibition of ligand binding to EGFR by certain mAbs effectively suppresses tumor growth in vitro and in vivo (5, 12, 13). Clinical trials of these antibodies in patients with squamous cell carcinoma of the head and neck or the lung (13) or with malignant gliomas (14) have yielded encouraging results.

The EGFR gene is amplified in nearly 50% of human grade IV gliomas (glioblastoma multiforme; Refs. 4, 15). In the majority of these cases, amplification of the EGFR gene is associated with structural rearrangements of the gene. The most frequent mutation has been observed in more than 25% of gliomas (15–17) and, also reported in carcinomas of the lung, breast, and prostate (18–20), results from the deletion of the coding exons 2–7, which leads to an in-frame deletion of 801 bp from the mature mRNA. This corresponds to a deletion of 267 amino acids from the NH2 terminus of the EGFR and creates a mutant EGFR with a unique extracellular domain (15). The mutant ΔEGFR is ligand independent and constitutively active, and its expression in human glioma cells enhances tumorigenicity (21, 22) and increases the invasive phenotype of the cell in vivo (23). The ΔEGFR-selective tyrosine kinase inhibitor, AG1478, effectively suppresses the growth of ΔEGFR-overexpressing glioblastoma cells (24), which suggests that the blockade of ΔEGFR activation can inhibit the aggressive phenotype of glioblastomas. Correspondingly, the presence of ΔEGFR in tumors correlates with poor prognosis in glioma patients (25). The oncogenic potential mediated by ΔEGFR together with its high level and restricted expression in tumor tissue, as well as its localization on the cell surface, make this mutant EGFR a potential target for antibody-based therapy.

Here we demonstrate that systemic treatment with the novel ΔEGFR-specific mAb, mAb 806, causes reduced phosphorylation of the constitutively active ΔEGFR and thereby suppresses growth of intracranially implanted gliomas overexpressing this mutant receptor in nude mice and extends their survival. The inhibition of tumor growth was mediated by a decrease in proliferation and angiogenesis and increased apoptosis of the tumor cells. This suppression activated active signaling by ΔEGFR because intracranial xenografts that were derived from cells overexpressing kinase-deficient ΔEGFR (DK), which are recognized equally well by mAb 806, were not significantly
suppressed after the same therapy. These results suggest that ΔEGFR may be a useful tumor-specific target for antibody-mediated therapy.

Materials and Methods

Cell Lines. Because primary explants of human glioblastomas rapidly lose expression of amplified, rearranged receptors in culture, no existing glioblastoma cell lines exhibit such expression. To force maintenance of expression levels comparable with those seen in human tumors, U87 MG, LN-Z308, and A1207 (gift from Dr. S. Aaronson, Mount Sinai Medical Center, New York, NY) were infected with ΔEGFR, kinase-deficient ΔEGFR (DK), or ΔwEGFR viruses which also conferred resistance to G418 as described previously (21). Populations expressing similar levels of the various EGFR alleles (these expression levels correspond approximately to an amplification level of 25 gene copies; human glioblastomas typically have amplification levels from 10 to 50 gene copies of the truncated receptor) were selected by FACs as described previously (21) and designated as U87 MG.ΔEGFR, U87 MG.DK, U87 MG.ΔwEGFR, LN-Z308.ΔEGFR, LN-Z308.DK, LN-Z308.ΔwEGFR, A1207.ΔEGFR, A1207.DK, and A1207.ΔwEGFR, respectively. Each was maintained in medium containing G418 (U87 MG cell lines, 400 μg/ml; LN-Z308 and A1207 cell lines, 800 μg/ml).

mAbs. mAb 806 (IgG2b, k), a ΔEGFR specific mAb, was produced after immunization of mice with NR6 mouse fibroblasts expressing the ΔEGFR. It was selected from several clones because hemagglutination assays showed that it had a high reactivity against NR6.ΔEGFR cells, low reactivity for NR6.ΔwEGFR cells, and none for NR6 cells.

 Immunoprecipitation and Western Blot Analysis. Cells were lysed with lysis buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 0.1% SDS, 0.5% sodium deoxycholate, 10 mM sodium PP, 1 mM phenylmethylsulfonyl fluoride, 2 mM Na3VO4, 5 μg/ml leupeptin, and 5 μg/ml aprotinin. Antibodies were incubated with cell lysates at 4°C for 1 h before the addition of protein-A and -G Sepharose. Immunoprecipitates were washed twice with lysis buffer and once with HNTG buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol), electrophoresed, and transferred to nitrocellulose membranes. Blots were probed with the anti-EGFR antibody, C13, and proteins were visualized using the ECL chemiluminescent detection system (Amersham Pharmacia Biotech.). The mAbs used for precipitation were mAb 806, anti-EGFR mAb clone 528 (Oncogene Research Products, Boston, MA), or clone EGFR-1 (Oncogene Research Products). A mAb, C13, used for detection of both wild-type and ΔEGFR on immunoblots was provided by Dr. G. N. Gill (University of California, San Diego, CA). Antibodies to Bcl-X (rabbit polyclonal antibody; Transduction Laboratories, Lexington, KY) and phosphotyrosine (4G10, Upstate Biotechnology, Lake Placid, NY) were used for Western blot analysis as described previously (26).

 Flow Cytometry Analysis. Cells were labeled with the relevant antibody followed by fluorescein-conjugated goat anti-mouse IgG (1:100 dilution; Becton-Dickinson PharMingen, San Diego, CA) as described previously (21). Stained cells were analyzed with a FACSCalibur using Cell Quest software (Becton-Dickinson PharMingen). For the first antibody, the following mAbs were used: mAb 806, anti-EGFR mAb clone 528, and clone EGFR-1. Mouse IgG2a or IgG2b was used as an isotype control.

Tumor Therapy. U87 MG.ΔEGFR cells (1 × 105) or 5 × 105 LN-Z308.ΔEGFR, A1207.ΔEGFR, U87 MG, U87 MG.DK, and U87 MG.ΔwEGFR cells in 5 μl of PBS were implanted into the right corpus striatum of nude mice as described previously (27). Systemic therapy with mAb 806, or the IgG2b isotype control, was accomplished by i.p. injection of 1 mg of mAbs in a volume of 100 μl every other day from postimplantation day 0 through 14. For direct therapy of intracerebral U87 MG.ΔEGFR tumors, 10 μg of mAb 806, or the IgG2b isotype control, in a volume of 5 μl were injected at the tumor-injection site every other day starting at day 1 for 5 days.

 Immunohistochemistry. To assess angiogenesis in tumors, they were fixed in a solution containing zinc chloride, paraffin embedded, sectioned, and immunostained using a monoclonal rat antimouse CD31 antibody (Becton-Dickinson PharMingen; 1:200). Assessment of tumor cell proliferation was performed by Ki-67 immunohistochemistry on formalin-fixed paraffin-embedded tissue sections. After deparaffinization and rehydration, the tissue sections were incubated with 3% hydrogen peroxide in methanol to quench endogenous peroxidase. The sections were blocked for 30 min with goat serum and incubated overnight with the primary antibody at 4°C. The sections were then washed with PBS and incubated with a biotinylated secondary antibody for 30 min. After several washes with PBS, products were visualized using streptavidin horseradish peroxidase with diaminobenzidine as chromogen and hematoxylin as the counterstain. As a measure of proliferation, the Ki-67 labeling index was determined as the ratio of labeled:total nuclei in high-power (×400) fields. Approximately 2000 nuclei were counted in each case by systematic random sampling. For macrophage and NK cell staining, frozen sections, fixed with buffered 4% paraformaldehyde solution, were immunostained using biotinylated mAb F4/80 (Serotec, Raleigh, NC) and polyclonal rabbit anti-asialo GM1 antibody (Dako Chemicals, Richmond, VA), respectively. Angiogenesis was quantitated as vessel area using computerized analysis. For this purpose, sections were immunostained using anti-CD31 and were analyzed using a computerized image analysis system without counterstain. MVAs were determined by capturing digital images of the sections at ×200 magnification using a CCD color camera as described previously (27). Images were then analyzed using Image Pro Plus version 4.0 software (Media Cybernetics, Silver Spring, MD) and MVA was determined by measuring the total amount of staining in each section. Four fields were evaluated for each slide. This value was represented as a percentage of the total area in each field. Results were confirmed in each experiment by at least two observers (K. M., H.-J. S. H.).

TUNEL Assay. Apoptotic cells in tumor tissue were detected by using the TUNEL method as described previously (27). TUNEL-positive cells were counted at ×400. The apoptotic index was calculated as a ratio of apoptotic cell number:total cell number in each field.

Statistical Analysis. The data were analyzed for significance by Student’s t test, except for the in vivo survival assays, which were analyzed by Wilcoxon analysis.

Results

Systemic Treatment of mAb 806 Extends the Survival of Mice Bearing ΔEGFR-overexpressing Intracranial Glioma Tumors. To test the efficacy of the anti-ΔEGFR mAb, mAb 806, we treated nude mice bearing intracranial ΔEGFR-overexpressing glioma xenografts with i.p. injections of mAb 806, the isotype control IgG, or PBS. U87 MG.ΔEGFR cells were implanted intracranially into nude mice, and the treatments began on the same day as described in “Materials and Methods.” Animals treated with PBS or isotype control IgG had a median survival of 13 days, whereas mice treated with mAb 806 had a 61.5% increase in median survival up to 21 days (P < 0.001; Fig. 1A). Treatment of mice 3 days postimplantation, after tumor establishment, also extended the median survival of the mAb 806-treated animals by 46.1% (from 13 days to 19 days; P < 0.01) compared with that of the control groups (data not shown). To determine whether these antitumor effects of mAb 806 extended beyond U87 MG.ΔEGFR xenografts, we also did similar treatments of animals bearing other glioma cell xenografts of LN-Z308.ΔEGFR and A1207.ΔEGFR. The median survival of mAb 806-treated mice bearing LN-Z308.ΔEGFR xenografts was extended from 19 days for controls to 58 days (P < 0.001; Fig. 1B). Remarkably, four of eight mAb 806-treated animals survived beyond 60 days (Fig. 1B). The median survival of animals bearing A1207.ΔEGFR xenografts was also extended from 24 days for controls to 29 days (P < 0.01; data not shown).

mAb 806 Treatment Inhibits ΔEGFR-overexpressing Brain Tumor Growth. Mice bearing U87 MG.ΔEGFR and LN-Z308.ΔEGFR xenografts were killed at day 9 and day 15, respectively. Tumor sections were histopathologically analyzed, and tumor volumes were determined as described in “Materials and Methods.” Consistent with the results observed for animal survival, mAb 806 treatment significantly reduced the volumes of U87 MG.ΔEGFR by ~90% (P < 0.001; Fig. 1C), and of LN-Z308.ΔEGFR by >95% (P < 0.001; Fig. 1D), of xenografts in comparison with those of the control groups. Similar results were obtained for animals bearing A1207.ΔEGFR tumors (65% volume reduction; P < 0.01; data not shown).
Intratumoral Treatment with mAb 806 Extends Survival of Mice Bearing U87 MG.ΔEGFR Brain Tumors. We also determined the efficacy of direct intratumoral injection of mAb 806 for the treatment of U87 MG.ΔEGFR xenografts. Animals were given intratumoral injections of mAb 806 or isotype control IgG at 1 day postimplantation, as described in “Materials and Methods.” Control animals survived for 15 days, whereas mAb 806 treated mice remained alive for 18 days (P < 0.01; Fig. 1E). Although the intratumoral treatment with mAb 806 was somewhat effective, it entailed the difficulties of multiple intracranial injections and of increased risk of infection. We, therefore, focused on systemic treatments for additional studies.

mAb 806 Treatment Slightly Extends Survival of Mice Bearing U87 MG.wtEGFR but not of Mice Bearing U87 MG or U87 MG.DK Intracranial Xenografts. To determine whether the growth inhibition by mAb 806 was selective for tumors expressing ΔEGFR, we treated animals bearing U87 MG, U87 MG.DK (kinase-deficient ΔEGFR) or U87 MG.wtEGFR brain xenografts. mAb 806 treatment did not extend the survival of mice implanted with U87 MG tumors (Fig. 2A), which expressed a low level of endogenous wtEGFR (22), or of animals bearing U87 MG.DK xenografts, which overexpressed a kinase-deficient ΔEGFR in addition to a low level of endogenous wtEGFR (22). The mAb 806 treatment slightly extended the survival of mice bearing U87 MG.wtEGFR tumors (P < 0.05; median survival, 23 days versus 26 days for the control groups), which overexpressed wtEGFR (Fig. 2B).

mAb 806 Reactivity Correlates with in Vivo Antitumor Efficacy. To understand the differential effect of mAb 806 on tumors expressing various levels or different types of EGFR, we determined mAb 806 reactivity with various tumor cells by FACS analysis. Consistent with previous reports (21), the anti-EGFR mAb 528 recognized both ΔEGFR and wtEGFR and demonstrated stronger staining for U87 MG.ΔEGFR cells compared with U87 MG cells (Fig. 3A, 528). In contrast, antibody EGFR.1 reacted with wtEGFR but not with ΔEGFR (21), because U87 MG.ΔEGFR cells were as weakly reactive as U87 MG cells (Fig. 3A, panel EGFR.1). This EGFR.1 antibody reacted with U87 MG.wtEGFR more intensively than with U87 MG cells, because U87 MG.wtEGFR cells overexpressed wtEGFR (Fig. 3A, panel EGFR.1). Although mAb 806 reacted intensely with U87 MG.ΔEGFR and U87 MG.DK cells and not with U87 MG cells, it reacted weakly with U87 MG.wtEGFR, which indicated that mAb 806 is selective for ΔEGFR with a weak cross-reactivity to overexpressed wtEGFR (Fig. 3A, panel mAb 806). This level of reactivity with U87 MG.wtEGFR was quantitatively and qualitatively similar to the extension of survival mediated by the antibody treatment (Fig. 2C).

We further determined mAb 806 specificity by immunoprecipitation. EGFRs in various cell lines were immunoprecipitated with antibody 528, EGFR.1, and mAb 806. Blocks of electrophoretically separated proteins were then probed with the anti-EGFR antibody, C13, which recognizes wtEGFR as well as ΔEGFR and DK (22). Consistent with the FACS analysis, antibody 528 recognized wtEGFR and mutant receptors (Fig. 3B–panel IP: 528), whereas antibody EGFR.1 reacted with wtEGFR but not with the mutant species (Fig. 3B, panel IP: EGFR.1). Moreover, the levels of mutant receptors in U87 MG.ΔEGFR and U87 MG.DK cells are comparable with those of

Fig. 1. A and B, extended survival of nude mice bearing intracranial U87 MG.ΔEGFR (A) and LN-Z308.ΔEGFR (B) xenografts with systemic mAb 806 treatment. U87 MG.ΔEGFR cells (1 × 10^6) or LN-Z308.ΔEGFR cells (5 × 10^6) were implanted into nude mice brains, and the animals were treated with either mAb 806, PBS, or isotype IgG from postimplantation days 0 through 14 as described in “Materials and Methods.” The mice were observed after discontinuation of therapy. Statistical significance was achieved by Wilcoxon analysis of Kaplan-Meier survival curves. ***P < 0.001 control versus mAb 806. Similar results were obtained in two independent experiments. C and D, growth inhibition of intracranial tumors by mAb 806 treatment. Nude mice (five per group), treated with either mAb 806 or the isotype control IgG, were euthanized on day 9 for U87 MG.ΔEGFR (C) and on day 15 for LN-Z308.ΔEGFR (D), and their brains were harvested, fixed, and sectioned. After H&E staining, tumor volumes were determined by using the diameters that were measured at the maximal brain tumor dimensions in coronal sections.

Data were calculated by taking the tumor volume of control as 100%. Values are mean ± SD. ***P < 0.001; control versus mAb 806. Arrowheads, tumor tissue. E, extended survival of nude mice bearing intracranial U87 MG.ΔEGFR xenografts with intratumoral mAb 806 treatment. U87 MG.ΔEGFR cells were implanted as described. Ten μg of mAb 806 or isotype IgG control in a volume of 5 μl were injected at the tumor-injection site every other day starting at day 1 for five times. Statistical significance was achieved by Wilcoxon analysis of a Kaplan-Meier survival curve. ***, P < 0.01; control versus mAb 806. Similar results were obtained in two independent experiments.
wtEGFR in the U87 MG.wtEGFR cells (Fig. 3B, panel IP: 528). However, antibody mAb 806 was able to precipitate only a small amount of the wtEGFR from the U87 MG.wtEGFR cell lysates as compared with the larger amount of mutant receptor precipitated from U87 MG.ΔEGFR and U87 MG.DK cells, and an undetectable amount from the U87 MG cells (Fig. 3B, panel IP: mAb 806). Collectively, these data suggest that mAb 806 recognizes an epitope in ΔEGFR that also exists in a small fraction of wtEGFR only when it is overexpressed on the cell surface.

**mAb 806 Treatment Reduces ΔEGFR Autophosphorylation and Down-Regulates Bcl-X<sub>L</sub> Expression in U87 MG.ΔEGFR Brain Tumors.** We next investigated the mechanisms underlying the growth inhibition by mAb 806. Because the constitutively active kinase activity and autophosphorylation of the COOH terminus of ΔEGFR are essential for its biological functions (21, 22, 28, 29), we determined ΔEGFR phosphorylation status in tumors from treated and control animals. As shown in Fig. 4A, mAb 806 treatment dramatically reduced ΔEGFR autophosphorylation, although receptor levels were only slightly decreased in the mAb 806-treated xenografts. We have previously shown that receptor autophosphorylation causes up-regulation of the antiapoptotic gene, Bcl-X<sub>L</sub>, which plays a key role in reducing apoptosis of ΔEGFR-overexpressing tumors (28, 29). Therefore, we next determined the effect of mAb 806 treatment on Bcl-X<sub>L</sub> expression. ΔEGFR tumors from mAb 806-treated animals did indeed show reduced levels of Bcl-X<sub>L</sub> (Fig. 4B).

**mAb 806 Treatment Decreases Growth and Angiogenesis and Increases Apoptosis in U87 MG.ΔEGFR Tumors.** In light of the in vivo suppression caused by mAb 806 treatment and its biochemical effects on receptor signaling, we determined the proliferation rate of tumors from control or treated mice. The proliferative index, measured by Ki-67 staining of the mAb 806-treated tumors, was significantly lower than that of the control tumors (P < 0.001; Fig. 5). In addition, analysis of the apoptotic index through TUNEL staining demonstrated a significant increase in the number of apoptotic cells in mAb 806-treated tumors as compared with the control tumors.

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Fig. 2. mAb 806 slightly extends survival of mice with U87 MG.wtEGFR brain tumors but not with U87 MG. DK, or U87 MG brain tumors. U87 MG (A), U87 MG. DK (B), or U87 MG.wtEGFR (C) cells (5 × 10<sup>5</sup>) were implanted into nude mice brains, and the animals were treated with mAb 806 from postimplantation days 0 through 14 as described in “Materials and Methods,” followed by observation after discontinuation of therapy. Statistical significance was achieved by Wilcoxon analysis of a Kaplan-Meier survival curve. *, P < 0.05; control versus mAb 806. Similar results were obtained in two independent experiments.

Fig. 3. A, FACS analysis of mAb 806 reactivity with U87 MG cell lines. U87 MG, U87 MG.ΔEGFR, U87 MG.DK, and U87 MG.wtEGFR cells were stained with anti-EGFR mAbs 528, EGFR.1, and anti-ΔEGFR antibody, mAb 806, as described in “Materials and Methods.” Monoclonal EGFR.1 antibody recognized wtEGFR exclusively and monoclonal 528 antibody reacted with both wtEGFR and ΔEGFR. mAb 806 reacted intensively with U87 MG.ΔEGFR and U87 MG. DK and weakly with U87 MG.wtEGFR. Bars on the abscissa, maximum staining of cells in the absence of primary antibody. Results were reproduced in three independent experiments.

B, mAb 806 immunoprecipitation of EGFR forms. Mutant and wtEGFR were immunoprecipitated with anti-EGFR antibodies, 528, or EGFR.1, or anti-ΔEGFR antibody, mAb 806, from (Lane 1) U87 MG, (Lane 2) U87Δ.EGFR, (Lane 3) U87 MG. DK, and (Lane 4) U87 MG.wtEGFR cells and were then detected by Western blotting with anti-pan EGFR antibody, C13, as described in “Materials and Methods.” Results were reproduced in three independent experiments.
Fig. 4. Systemic treatment with mAb 806 decreases the phosphorylation of ΔEGFR and Bcl-XL expression in U87 MG.ΔEGFR brain tumors. U87 MG.ΔEGFR tumors were resected at day 9 of mAb 806 treatment, immediately frozen in liquid nitrogen and stored at −80°C before tumor lysate preparation. A, Western blot analysis of expression and the degree of autophosphorylation of ΔEGFR. Thirty μg of tumor lysates were subjected to SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and probed with antiphosphotyrosine mAb, then were stripped and reprobed with anti-EGFR antibody, C13. B, Western blotting of Bcl-XL by using the same tumor lysates as in A. Membranes were probed with antihuman Bcl-XL polyclonal antibody. Lanes 1 and 2, U87 MG.ΔEGFR brain tumors treated with isotype control; Lanes 3 and 4, U87 MG.ΔEGFR brain tumors treated with mAb 806.

(P < 0.001; Fig. 5). The extent of tumor vascularization was also analyzed by immunostaining of tumors from treated and control specimens for CD31. To quantify tumor vascularization, MVAs were measured using computerized image analysis, mAb 806-treated tumors showed 30% less MVA than did control tumors (P < 0.001; Fig. 5). To understand whether interaction between receptor and antibody may elicit an inflammatory response, we stained tumor sections for the macrophage marker, F4/80, and the NK cell marker, asialo GM1. Macrophages were identified throughout the tumor matrix and especially accumulated around the mAb 806-treated-U87 MG.ΔEGFR-tumor periphery (Fig. 5). We observed few NK cells infiltrated in and around the tumors and no significant difference between mAb 806-treated and isotype-control tumors (data not shown).

Discussion

ΔEGFR appears to be an attractive potential therapeutic target for cancer treatment of gliomas. It is correlated with poor prognosis (25), whereas its genetic or pharmacological inhibition effectively suppresses growth of ΔEGFR-overexpressing cells both in vitro and in vivo (29, 30). Because this mutant EGFR is expressed on the cell surface, it represents a potential target for antibody-based therapy, and, here, we tested the efficacy of a novel anti-ΔEGFR mAb, mAb 806, on the treatment of intracranial xenografts of ΔEGFR-overexpressing gliomas of different cellular backgrounds in nude mice. We showed that the systemic administration of mAb 806 inhibited tumor growth and extended animal survival. The effect of mAb 806 was evident for each cell line and was independent of the p53 status of the tumors, because U87 MG.ΔEGFR and A1207.ΔEGFR expressed wild-type p53, whereas LN-Z308.ΔEGFR was p53-null.

The enhanced tumorigenicity of ΔEGFR is mediated through its constitutively active kinase activity and tyrosine autophosphorylation at the COOH terminus (22, 28, 29). Phosphorylation of ΔEGFR in mAb 806-treated tumors was significantly decreased, proliferation was reduced, and apoptosis was elevated, which suggests that the antitumor effect of mAb 806 is, at least in part, attributable to the inhibition of the intrinsic function of the receptor. The ΔEGFR signaling caused up-regulation of the antiapoptotic gene, Bcl-XL (28), and treatment with mAb 806 resulted in down-regulation of Bcl-XL expression, which further suggests that the antitumor effect of mAb 806 is mediated through the inhibition of ΔEGFR signaling. The level of ΔEGFR in the mAb 806-treated tumors was also slightly reduced (Fig. 4A), but not to a degree that was consistent with the degree of dephosphorylation of the mutant receptor or sufficient to explain the magnitude of its biological effect. The antitumor effect of mAb 806 is likely to result, at least in part, from the inhibition of the intrinsic signaling function of ΔEGFR. This assertion is also supported by the lack of antitumor effects on DK tumors, which bind to the antibody but are kinase deficient.

Intratumoral injection of a different anti-ΔEGFR antibody, mAbY10, inhibited the growth of ΔEGFR-expressing B16 melanoma tumors in mouse brains through a Fc/Fc receptor-dependent mechanism (31). In conjunction with this, mAbY10 was shown to mediate antibody-dependent macrophage cytotoxicity in vitro with both murine and human effector cells (17), although it had little effect with
systemic administration, in contrast to mAb 806. Intensive macrophage infiltration found in our mAb 806-treated tumors raises the question as to whether the antitumor effect of mAb 806 may be accomplished by macrophage-mediated cytotoxicity. We believe this to be unlikely, because macrophage infiltration also occurred on mAb 806 treatment of U87 MG.DK (kinase-deficient EGFR) tumors, in which it was ineffective in regulating tumor growth.

mAb 806 appears to be selective for EGFR with a weak cross-reactivity with overexpressed wtEGFR. Consistent with the in vitro specificity, mAb 806 treatment was very effective in EGFR-overexpressed tumors, whereas it showed a much less robust, but reproducible, growth inhibition for tumors overexpressing wtEGFR. However, the simple interaction between mAb 806 and its target molecules is insufficient to inhibit tumor growth because, although mAb 806 is capable of binding equally well to kinase-deficient EGFR (DK) receptors and EGFR, it is ineffective in affecting DK-expressing tumor growth. The inability of mAb 806 to interact with the low-level EGFR-overexpressed tumors when compared with normal tissues.

Although the mAb 806 treatment was effective for suppression of intracranial xenografts, it should be noted that the EGFR-tumors eventually grew, and durable remissions were not achieved. This may have resulted from inefficient distribution of antibody in the tumor mass. mAbs in combination with other therapeutic modalities such as toxins, isotopes or drugs, for cancer treatments have been shown to be more effective than antibody alone in many cases (2, 3, 32–34).

Combination treatments targeted at tumor growth as well as angiogenic development have more effectively inhibited glioblastoma growth than either treatment alone (27). This raises the possibility that mAb 806 in combination with chemotherapeutic drugs or compounds modulating angiogenesis may be even more effective than mAb 806 alone.

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Chemotherapeutic drugs such as doxorubicin and cisplatin in conjunction with wtEGFR antibodies have also shown enhanced antitumor activity (35, 36).

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