Amplification and Expression of the Epidermal Growth Factor Receptor Gene in Human Glioma Xenografts

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ABSTRACT

Xenografts from eight malignant human gliomas were established in athymic mice and were used to study amplification and expression of the epidermal growth factor receptor (EGFR) gene. Tissue identity between biopsy and xenografts was confirmed by karyotypic profiles, which showed that each glioma xenograft retained structural abnormalities, including double minute chromosomes, present in the parent glioma. EGFR gene amplification was found in six of the eight glioma biopsies and their corresponding xenografts. Expression of the EGFR gene was measured by Scatchard analysis, affinity reactions, immunoprecipitations, Western immunoblots, and immunocytochemistry; significant expression of the EGFR gene was only detectable in xenografts with EGFR gene amplification. Moreover, five of the six xenografts with EGFR gene amplification demonstrated structural alterations of the EGFR gene, which was associated with low-molecular-weight EGFR proteins. These xenografts represent an excellent tissue source and in vivo model system for characterizing the epidermal growth factor receptor in malignant human gliomas.

INTRODUCTION

The EGFR gene is often amplified and highly expressed in malignant human gliomas. In biopsy tissue, 4 of 10 or 40% (1) and 24 of 63 or 38% (2) of gliomas were found to have amplified the EGFR gene, and this amplification was associated with increased receptor kinase activity (1) and increased mRNA levels as assessed by in situ hybridization (2). Most gliomas with gene amplification have been demonstrated to contain extrachromosomal DMs, which presumably contain the amplified genes (3). Rearrangement of the EGFR gene has been sometimes reported to accompany its amplification. In the squamous carcinoma cell line A431, for example, an amplified and rearranged EGFR gene is associated with a variant 2.9-kilobase mRNA (4) and a carboxy-truncated and secreted 105-kDa protein (5). Rearrangement of the EGFR gene in gliomas has been observed in several cases (1, 6), but an effect of this rearrangement on the gene product, if any, has not been demonstrated.

The amplification and high expression of the EGFR gene in human gliomas may have significant consequences for the growth of these tumors. Study of this gene in gliomas has, however, been hampered by the fact that biopsies of the primary tumors often consist of minute quantities of tissue, thereby presenting logistical difficulties to detailed molecular and functional studies. Moreover, there is only one human glioma-derived cell line which is reported to retain amplification of the EGFR gene in culture (6); evidently the selective conditions operating in vivo to maintain the amplified EGFR gene are not present during in vitro cultivation.

In this study we report the establishment in athymic mice of 8 serially transplantable subcutaneous xenografts derived from malignant gliomas. The xenografts maintained many of the properties of the primary tumors from which they were derived, including karyotypic abnormalities and the presence of gene amplification. Six of the 8 xenografts contained an amplified EGFR gene, and 5 of those with amplification exhibited structural alterations of the EGFR gene. One glioma amplified a rearranged EGFR gene that expressed a small EGFR protein in some ways resembling the viral erbB oncogene protein; it did not bind EGF and did not react with antibodies to the EGFR extracellular domain but did react with an antibody reactive with the intracytoplasmic portion of the EGFR. These xenografts represent an in vivo model system for studying gene amplification in malignant human gliomas, and they provide an excellent tissue source for purification and characterization of the products of the amplified genes.

MATERIALS AND METHODS

Growth of Human Glioma Xenografts in Athymic Mice. Glioma samples obtained at surgery were sterilely sectioned into pieces allowing s.c. inoculation through a 16-gauge needle into the right flank of recipient athymic mice. Athymic BALB/c mice (nu/nu genotype, male or female, 6 wk or older) were used for the growth of all xenografts as described (7). Tumor transplantation s.c. was performed as described previously (8).

Analysis of Amplification by Hybridization. DNA was purified from frozen biopsies of eight malignant human gliomas and from frozen xenograft tissue derived from these eight biopsies using methods detailed previously (9). DNA (1.5 to 3 µg) was cleaved with EcoRI, separated by electrophoresis through a 1% agarose gel, blotted onto a nylon membrane, and hybridized with an EGFR probe labeled with [32P]dCTP via oligolabeling. The EGFR probe used was the 1.6-kilobase EcoRI fragment of pE7 (10), a cDNA clone of EGFR mRNA generously provided by Dr. G. Merlino and Dr. I. Pastan. Washing of filters and autoradiography were as described (9). The signals were removed by boiling in H2O for 5 min, and the filters were rehybridized with the 1.0-kilobase EcoRI/BamHI fragment of pNB-1 (11) containing part of the second exon of the N-myc gene to control for DNA loading. The amplification level was determined by comparative densitometry of the two sets of autoradiographs.

Chromosomal Analyses. Karyotypes of the human biopsies from which the xenografts were derived have been published previously (12-14). Xenograft tissue was dissected aseptically, finely minced, and either enzymatically dissociated using Hanks' balanced salts solution (pH 7.0) containing 0.2% collagenase (125 units/mg) (Sigma), 0.05% Pronase (Sigma) 4 units/mg, B grade), and 0.02% DNase (Sigma) (7 × 10³ dornase units/mg of DNase I, B grade) at 37°C for approximately 45 min or incubated for 4 to 16 h in serum-free zinc option medium

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; DMs, double minute chromosomes; kDa, kilodaltons; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazinenucilaminic acid; BSA, bovine serum albumin; cDNA, complementary DNA; ED₅₀, the effective dose of unlabeled EGF required to specifically displace 50% of [¹²⁵I]-EGF.

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; cDNA, complementary DNA; I'D,,,, the effective dose of unlabeled EGF required

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EGF receptors in gliomas

containing 8 mg/ml of collagenase at 37°C. These cell suspensions were centrifuged, and the pellet was resuspended in zinc option medium containing 10% fetal calf serum. Cell counts and trypsin blue viability counts were done, and cells were plated in two or four 100-mm dishes at 5 × 10^5 viable cells per dish. Following 48- to 96-h incubation at 37°C in a 5% CO₂ atmosphere, cholines were added to a final concentration of 0.1 μg/ml, and these dishes were incubated at 37°C in 5% CO₂ for 1 h. Chromosomal preparations were made, and Giemsa-trypsin-banded slides were prepared using previously published procedures (12).

Modal numbers were determined by counting at least 25 metaphases per preparation. Karyotypes were determined by arranging all photographed metaphases that were technically satisfactory according to the International System for Human Cytogenetics Nomenclature (15). The most frequent chromosomal constitution of each preparation was designated the stem line.

Immunocytochemistry. Human gliomas grown in athymic mice were harvested and snap frozen at −80°C, then cut into 6-μm-thick sections onto glass slides coated with Histostik (Accurate Chemical Co.), and fixed in −20°C acetone for 1 min. Fixation and all the following incubations were followed by 0.115 M phosphate buffer, pH 7.4, washes unless otherwise noted. Endogenous peroxidase activity was blocked by a 20-min incubation of 0.3% H₂O₂ in methanol; nonspecific protein binding was blocked by incubation in 10% normal goat serum for 30 min. The 10% normal goat serum was aspirated without washing, and the slides were incubated overnight in either rabbit anti-EGF receptor serum No. 451 (16) at a 1:4,000 dilution or normal rabbit serum (1:4,000 dilution) (both generous gifts from Dr. Christa Stoscheck). Polyclonal antibody 451 is reactive with the extracellular EGFR domain. Secondary antibody consisted of biotinylated goat anti-rabbit antibody (1:200 dilution) for a 30-min incubation, followed by a 30-min incubation with an avidin and biotinylated peroxidase solution made according to the manufacturer’s instructions (Vector Laboratories, Burlingame, CA). The chromagen was diaminobenzidine (66.7 mg/dl in 0.115 M phosphate buffer solution with 0.006% H₂O₂, 6-min incubation). The slides were counterstained with hematoxylin, dehydrated through graded alcohols and xylene, and then mounted with Permount (Fisher Scientific Co.) and glass coverslips. Positive controls consisted of normal human skin sections, which exhibited staining for the EGFR receptor as has been previously described (17). Sections demonstrating reactivity were scored (+), sections without reactivity were scored (−), and equivocal staining was scored (+/−).

Preparation of Membranes from Xenograft Tissue. Microsomal membranes were prepared as described (18), with modifications. Tumors were harvested and immediately placed in a cold (4°C) 0.3 M sucrose solution. The tissue was cut into small fragments and homogenized for 5 min in a Potter-Elvehjem homogenizer with a stirring motor. The homogenate was centrifuged at 15,000 × g for 20 min, and the supernatant was used in EGFR immunoprecipitation. Immunoprecipitation was performed with either monoclonal antibody Ab-1 (clone 528; Oncogene Science, Inc.), reactive against the extracellular EGFR domain, or polyclonal antibody 2913, reactive against the intracytoplasmic portion of the EGFR (24) and generously provided by Dr. I. Pastan, NIH. Monoclonal antibody Ab-1 (528) is an IgG2a which inhibits EGF binding to its receptor (25–28), while polyclonal antibody 2913 specifically recognizes the cytoplasmic portion of the EGFR in A431 carcinoma cells, in normal human fibroblasts, and in a variety of other human tumor cell lines (29). Antibody 2913 is reactive with the viral erbB protein, which is predominantly intracytoplasmic but does not react with the carboxy-truncated and secreted 105 kDa EGFR variant, which is related to the cell surface EGFR domain (24). For each reaction mixture, 5 μg of monoclonal antibody Ab-1 (528) or 15 μl of undiluted antisera containing polyclonal antibody 2913 were bound to 2 mg of Protein A-Sepharose 4B (Sigma) by incubation in 115 mM sodium phosphate buffer, pH 7.4, for 30 min at room temperature. Antibody-Protein A-Sepharose complex was washed 3 times with 115 mM sodium phosphate buffer, pH 7.4. EGFR immunoprecipitation was performed with 500-μl aliquots of solubilized xenograft tissue, 500 μl of 115 mM sodium phosphate buffer, pH 7.4, and the antibody-Protein A-Sepharose pellet.

Table 1 Stem-line karyotypes of human glioma xenografts

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Stem-line karyotype*</th>
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<tr>
<td>D-320 MG</td>
<td>52,XX,−9,+13,+14,+17,+20,+21,+22,+del(5)(q15−21), +del(7)(p12), del(22) (q12), der(3)(2;14)(p25;q13)</td>
</tr>
<tr>
<td>D-256 MG</td>
<td>46,XY,+6,+7,+8,+22,r(9;19)(p13q11), der(9)(69p11p13), +DMs</td>
</tr>
<tr>
<td>D-270 MG</td>
<td>46,XY,+6,+7,+DMs</td>
</tr>
<tr>
<td>D-298 MG</td>
<td>45,XY,+10,+17,+20,del(1p22),del(5q34), del(22)(q7)p61p21p12, +der(17)(7q12p13q22), del(14)(q21p12q23)</td>
</tr>
<tr>
<td>D-317 MG</td>
<td>90,XYXY,−5,−7,−7,−7,−10,+10,+12,+12−14, −14−22,−22,del(9p21), del(9p21)(t1;19)(p32q32), t(10;19)p32q32, dup(9)(p11p12), der(15)(15;7q13q32), der(15)(15;7q22q11)</td>
</tr>
<tr>
<td>D-303 MG</td>
<td>79,XXX,−1,−2,−3,−4,−5,−7,−7,−10,+10,+12,+12−14, −14−21,−21,−22,del(3p11), del(3p11), +del(3) (p11), del(15)(q23), del(21)(3;21)(q11p13), +DMs</td>
</tr>
<tr>
<td>D-274 MG</td>
<td>64,XXX,−3,−3,−4,−4,−5,−6,−6,−10,+11,+12, −12−13,−13−14,−14−15,−16−16−17−17, −18−19−19−21−22,−22, del(9p13), del(9p13), t(1;19)(p32q21), t(1;19)(p32q21), del(13)(13;13)</td>
</tr>
<tr>
<td>D-245 MG</td>
<td>55,XY,+1,+5,−7,+8,+18,+19,+20,+22,15(15;17)</td>
</tr>
</tbody>
</table>

* Stem-line karyotype using Giemsa-trypsin banding expressed as recommended by the International System for Human Cytogenetics Nomenclature (15).

EGF receptors in gliomas

EGWF filters using a multiple manifold apparatus. The filters were washed with 3 ml of cold 20 mM HEPES buffer, pH 7.4, with 0.1% BSA, and the amount of radioactivity retained on each filter was counted in a Packard Auto-Gamma spectrometer. The binding data were analyzed by the Scatchard method on an IBM PC/AT computer using the EBDA software program (equilibrium binding data analysis), written by G. A. McPherson (21, 22) and obtained from Biomedical Computing Technology Information Center, R-1302, Vanderbilt University Medical Center, Nashville, TN.

Immunoprecipitation of EGFR. EGFR from xenograft tissue was solubilized and immunoprecipitated as described (23), with modifications. Ten mg of frozen (−70°C) xenograft tissue were homogenized in 1 ml of ice-cold solubilization buffer composed of 20 mM HEPES, pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 4 mM iodoacetate (Aldrich), and Aprotinin (Sigma) at 1 mg/ml. After 2 h at 4°C, the preparation was centrifuged for 15 min at 4°C in a Brinkman tabletop centrifuge at 12,000 × g. The supernatant was used in EGFR immunoprecipitation. Immunoprecipitation was performed with either monoclonal antibody Ab-1 (clone 528; Oncogene Science, Inc.), reactive against the external EGFR domain, or polyclonal antibody 2913, reactive against the intracytoplasmic portion of the EGFR (24) and generously provided by Dr. I. Pastan, NIH. Monoclonal antibody Ab-1 (528) is an IgG2a which inhibits EGF binding to its receptor (25–28), while polyclonal antibody 2913 specifically recognizes the cytoplasmic portion of the EGFR in A431 carcinoma cells, in normal human fibroblasts, and in a variety of other human tumor cell lines (29). Antibody 2913 is reactive with the viral erbB protein, which is predominantly intracytoplasmic but does not react with the carboxy-truncated and secreted 105 kDa EGFR variant, which is related to the cell surface EGFR domain (24). For each reaction mixture, 5 μg of monoclonal antibody Ab-1 (528) or 15 μl of undiluted antisera containing polyclonal antibody 2913 were bound to 2 mg of Protein A-Sepharose 4B (Sigma) by incubation in 115 mM sodium phosphate buffer, pH 7.4, for 30 min at room temperature. Antibody-Protein A-Sepharose complex was washed 3 times with 115 mM sodium phosphate buffer, pH 7.4. EGFR immunoprecipitation was performed with 500-μl aliquots of solubilized xenograft tissue, 500 μl of 115 mM sodium phosphate buffer, pH 7.4, and the antibody-Protein A-Sepharose pellet.
EGF RECEPTORS IN GLIOMAS

Fig. 1. Southern blot depicting EGFR sequence amplification in glioma biopsies and xenografts. Three µg of DNA from either the surgical biopsy (B) or mouse xenograft (X) of the indicated glioma were digested with EcoRI, separated by electrophoresis, and subjected to Southern transfer as described in “Materials and Methods.” The blots were probed with a 1.6-kilobase EcoRI fragment from the EGFR cDNA clone pE7. The 8 fragments identified in DNA from normal lymphocytes (N), shown at the margins. The alterations in EGFR gene structure which can be seen in this figure are as follows: in D-245, only 2 of the 8 normal fragments (8.0 and 2.5 kilobases) were amplified; in addition, D-245 exhibited an aberrant 1.6-kilobase fragment. In tumor D-256, the normal 8.0-kilobase fragment was not amplified, and an aberrant 7.0-kilobase fragment was present; DNA degradation in the surgical biopsy diminished the signal of the larger fragments. In tumor D-270, the 6.8- and 5.8-kilobase fragments were not amplified, but an aberrant 3.0-kilobase fragment was present. Tumors D-274 and D-303 exhibited all 8 normal fragments at normal intensity. In tumor D-298, neither the 1.8-kilobase fragment nor the 8.0-kilobase fragment was amplified; a rearranged 4.5-kilobase fragment could be seen in longer exposures. In the xenograft sample from D-317, the 6.8- and 5.8-kilobase fragments were not amplified, though these fragments could be seen in the original biopsy sample of the tumor. It is not known whether these differences between D-317 biopsy and xenograft were due to DNA degradation in the biopsy compounded by admixture with nonneoplastic cells, or due to a real structural difference between the amplified EGFR genes in biopsy versus xenograft. Tumor D-320 exhibited amplification of all 8 normal fragments detectable with the probe. The lanes containing samples in which EGFR genes were not amplified (N, D-274, and D-303) were exposed for 24 h. The other lanes, containing DNA samples with amplified EGFR genes, were exposed for 10 h. All exposures were at —70°C on Kodak XAR-5 film.

Autophosphorylation of EGFR. Autophosphorylation of the immunoprecipitated EGFR was performed as described (23). The EGFR-antibody-Protein A-Sepharose pellets were incubated with 30 µl of solubilization buffer plus 2 mM MnCl2 and 3 µCi of γ[32P]ATP (New England Nuclear; 2000 to 3000 Ci/mmol). After reaction for 10 min at 4°C on ice, the reaction was terminated by the addition of 30 µl of 2× Laemmli SDS-PAGE sample buffer with 2% β-mercaptoethanol. Samples were boiled for 3 min and centrifuged. Supernatants were used in loading SDS-polyacrylamide gels.

Immunoblot Analysis. Western immunoblot analysis was performed with the use of a semidry horizontal electrophoretic transfer system with graphite electrodes (LKB Multiphor II Nova Blot System). Briefly, frozen A431 and glioma xenografts were solubilized in SDS-gel sample buffer, boiled, and electrophoresed in a 7.5% SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose at 150 mA for 2 h, and immunohistochemical detection of EGFR was accomplished with antibody 2913 and the avidin-biotin complex method.

SDS-PAGE and Autoradiography. The SDS-discontinuous buffer system of Laemmli (30) with a 5% or 7.5% resolving gel was used. The high-molecular-weight standard mixture of myosin, β-galactosidase, phosphorylase b, bovine serum albumin, ovalbumin, and carbonic anhydrase (Sigma) was used to determine apparent molecular weights of the immunoprecipitated EGFR. Gels were stained with Coomassie Blue R250, destained, and dried under vacuum. Autoradiography was performed by exposing Kodak AR-5 film for varying lengths of time (1 to 12 h) at —70°C.

Phosphoamino Acid Analysis. The immunoprecipitated and phosphorylated D-245 MG EGFR was subjected to phosphoamino acid analysis as described (31). Briefly, 32P-EGFR was dissociated from Protein A-Sepharose and polyclonal antibody 2913 by 10% acetic acid, dried, and hydrolyzed with 6 M HCl. The hydrolysate was dried and resuspended in a solution of 2 mg of phosphoserine, phosphothreonine, and phosphotyrosine per ml. Paper electrophoresis on Whatman 3MM paper was carried out at pH 3.5 (H2O/acetic acid/pyridine, 945:50:5) at 1000 V for 3 h, with cooling. Standards were visualized by ninhydrin staining and 32P-amino acids by autoradiography with Kodak AR-5 film for 24 h at —70°C.
RESULTS

Karyotypic Analysis of the Eight Human Glioma Xenografts. The stem-line karyotypes of all eight xenografts were similar to the stem lines seen in the original human biopsies from which they were derived. The five xenografts with near-diploid stem lines, and hypotetraploid tumor D-303 MG differed from the original biopsies only in gains or losses of a small number of whole chromosomes. The remaining two xenografts with near-triploid or near-tetraploid stem lines were closely related to doubled versions of the near-diploid stem lines seen in the original human biopsies. Each tumor and derived xenograft except D-270 MG contained distinctive structural abnormalities (Table 1) which established their identity with certainty. DMs were present in both the original biopsy and xenograft in six of the eight cases. In D-320 MG and D-274 MG, DMs were not seen either originally or after transplantation.

Amplification of the EGFR Gene in Human Glioma Xenografts. Fig. 1 shows the results of hybridization of DNA purified from freshly frozen tumor tissue from 8 biopsies and corresponding xenografts with an EGFR cDNA probe. EGFR gene amplification (6- to 25-fold) was identified in 6 of the 8 glioma biopsies and in the xenografts derived from these 6 biopsies. The degree of amplification was quantitated by rehybridization of the same filters to a different probe (see "Materials and Methods"). The restriction pattern of the amplified EGFR gene indicated that, in 5 of the 6 xenografts with amplification, gene rearrangement had occurred, as characterized by the absence of normal fragments and/or the presence of abnormally migrating bands. For example, xenograft D-245 had no amplified the 6.8-, 5.8-, 2.0-, 1.8-, 1.5-, or 1.2-kilobase fragments but contained an abnormally migrating amplified 1.6-kilobase fragment. Similarly, xenograft D-270 had not amplified the 6.8- and 5.8-kilobase fragments but contained an abnormally migrating 3.0-kilobase segment. Other examples of gene alterations associated with amplification in the xenografts are described in the legend of Fig. 1. The same restriction pattern was observed in the xenograft and biopsy with the exception of D-317 (see Fig. 1 legend).

Detection of Glioma Xenograft EGFR Protein Expression by Immunohistochemistry. The polyclonal antibody 451, which is reactive with the extracellular domain of EGFR, bound to five of six of the glioma xenografts with the amplified EGFR gene (Table 2). Fig. 2 shows the immunostaining of the EGFR in a glioma with the amplified gene (D-320 MG) compared to a glioma without the amplified gene (D-274 MG). The one xenograft with amplified EGFR gene sequences which did not stain immunohistochemically was D-245 MG. EGFR immunoreactivity was identical in xenograft and parent biopsy tissue with the exception of D-274 MG, which was strongly positive for EGFR immunostaining in the biopsy but was negative in the xenograft tissue.

Quantitation of Glioma Xenograft EGFR Protein Expression by Scatchard Analysis of 125I-EGF Binding. Saturation curves of the direct and specific binding of increasing amounts of 125I-EGF to glioma membranes are shown in Fig. 3. Bmax values were computer generated and are presented in Table 2 as pmol of EGF bound per mg of membrane protein. Those gliomas with the amplified EGFR genes generally expressed larger numbers of EGFR (varying from 0.61 to 14.8 pmol/mg) com-
Fig. 3. Specific binding of increasing amounts of $^{125}$I-EGF to membranes from glioma xenografts D-320 MG (A), D-256 MG (O), D-270 MG (□), D-298 MG (△), D-317 MG (○), D-303 MG (□), D-274 MG (□), and D-245 MG (○). D-303, D-274, and D-245 MG had overlapping binding curves with negligible $^{125}$I-EGF binding, and all three are therefore indicated with a closed square. Membranes were incubated with $^{125}$I-EGF for 30 min at 25°C. Parallel incubation with at least a 100-fold excess of unlabeled EGF was used to determine nonspecific binding. Bound EGF was separated from free EGF by vacuum filtration over glass microfiber filters.

pared to glioma xenografts without the amplified gene (0.11 to 0.12 pmol/mg). The one exception was tumor D-245 MG, which bound very little EGF (0.07 pmol/mg), although amplification of the EGFR gene was detected. The xenograft with the highest level of EGFR (D-320 MG; Fig. 4A) expressed the protein at a level similar to that of xenograft tissue of A431 squamous carcinoma (15.0 pmol/mg). In culture, A431 cells express 2 to 3 x 10^6 EGFRs per cell, while most normal murine and human cells have 10^4 to 10^5 EGFRs per cell (32, 33). Three gliomas containing gene amplification (D-256 MG, D-270 MG, and D-298 MG) had levels of binding comparable to normal human placenta membranes (3.2 pmol/mg), while a fourth glioma with gene amplification (D-317 MG) bound fairly low amounts of growth factor. The very low binding capacity of the two xenografts without the amplified gene (D-274 MG and D-303 MG) was closer to normal brain (cerebrum), which did not specifically bind $^{125}$I-EGF.

Affinity of the EGF-Glioma EGFR Binding Reaction. As illustrated in Fig. 4B, the ED$_{50}$ was found to vary from 0.93 to 2.5 x 10^{-9} M. The ED$_{50}$ for EGF binding to A431 xenograft membranes was similar at 2.5 x 10^{-9} M. A second measure of EGF-glioma EGFR binding affinity was obtained by Scatchard analysis. The dissociation constant for EGF-glioma EGFR binding was similar to that for A431 and ranged from 1.5 to 7.7 x 10^{-9} M (Fig. 4A and data not shown).

Molecular Mass of Glioma EGFR. Fig. 5A exhibits the relative sizes of 32P-EGFR immunoprecipitated from gliomas with monoclonal antibody Ab-1 (528) to the extracellular domain of EGFR. The receptors from A431 and glioma D-320 MG were 170 kDa (Fig. 5A, Lanes 1 and 2); an accompanying 150-kDa proteolytic product was often observed. In contrast, a doublet of distinctly smaller-sized variant EGFRs of 120 to 145 kDa was present in the gliomas with amplified and rearranged EGFR genes (D-256 MG, D-270 MG, D-298 MG, and D-317 MG; Lanes 3 to 6, respectively). This result also demonstrated the autophosphorylating ability of the EGFR polypeptides from these xenografts since they were detected by their in vitro incorporation of 32P. The three gliomas with low EGFR levels by binding analysis (D-303 MG, D-274 MG, and D-245 MG) did not show immunoprecipitable 32P-EGFR bands (Fig. 5A, Lanes 7 to 9).

Immunoprecipitation and Western blot analysis were also performed with a polyclonal antibody (2913) reactive against the cytoplasmic portion of EGFR. The results with this antibody were indistinguishable from those obtained with monoclonal antibody Ab-1 (528) for the A431 xenograft as well as for the glioma xenografts, with the exception of D-245 MG (example in Fig. 5B). With D-245 MG, antibody 2913 immunoprecipitated an EGFR doublet of 80 and 100 kDa (Fig. 5B, Lane 2), while antibody Ab-1 (528) did not immunoprecipitate any detectable protein product (Fig. 5A, Lane 9). The autophosphorylating activity of the D-245 MG EGFR was demonstrated to be due to EGFR tyrosine kinase, as 32P-tyrosine was the predominant phosphoamino acid in immunoprecipitated and autophosphorylated D-245 MG EGFR by phosphoamino analysis (data not shown).

DISCUSSION

The data presented here demonstrate the successful establishment of transplantable subcutaneous xenografts from 8 malig-
EGF receptors in gliomas

Fig. 5. A, immunoprecipitation of EGFR from solubilized A431 and glioma xenograft homogenates with monoclonal antibody Ab-1 (528), which is reactive against the external EGFR domain. EGFRs in Triton X-100 solubilized xenograft homogenates from A431 (Lane 1) and glioma D-320 MG (Lane 2), D-256 MG (Lane 3), D-270 MG (Lane 4), D-298 MG (Lane 5), D-317 MG (Lane 6), D-274 MG (Lane 7), D-303 MG (Lane 8), and D-245 MG (Lane 9) were immunoprecipitated, autophosphorylated, and analyzed by SDS-PAGE with a 5% resolving gel. The gel was fixed and stained with Coomassie blue, destained, dried under vacuum, and exposed to X-ray film at -70°C for 3 h. Molecular weight standards are indicated on the left-hand margin (kDa). B, immunoprecipitation of EGFR from solubilized A431 and D-245 MG xenograft homogenates with antibody 2913 which is reactive against the EGFR cytoplasmic domain. EGFRs in Triton X-100 solubilized xenograft homogenates from A431 (Lane 1) and glioma D-245 MG (Lane 2) were immunoprecipitated, autophosphorylated with ³²P, and analyzed by 7.5% SDS-PAGE. Molecular weight standards are indicated on the left-hand margin (kDa).

Table 2 Human glioma xenograft EGFR gene amplification and expression

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Pathological diagnosis</th>
<th>Copies of EGFR gene[a]</th>
<th>Rmax (pmol/mg)[b]</th>
<th>Immunocytochemistry</th>
<th>Immunoprecipitation</th>
<th>Western immunoblot</th>
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<tr>
<td>D-320 MG</td>
<td>GBM</td>
<td>6</td>
<td>14.8</td>
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<td>D-256 MG</td>
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<td>3.1</td>
<td>+</td>
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<td>GBM</td>
<td>10</td>
<td>2.6</td>
<td>+</td>
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<td>+</td>
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<td>D-317 MG</td>
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<td>D-303 MG</td>
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<td>D-274 MG</td>
<td>GBM</td>
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<td>0.07</td>
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</table>

[a] Expressed as copies per haploid genome.
[b] Rmax determined by Scatchard analysis of direct, specific ³²P-EGF binding to tumor membranes. Units are pmol of ³²P-EGF bound per mg of membrane protein.
[c] GBM, glioblastoma multiforme; GS, gliosarcoma.
[d] Immunoprecipitation and Western blot analysis of EGFR were positive only with antibody 2913, reactive against the internal EGFR domain. No EGFR band was seen when antibody Ab-1 (528), reactive against the external portion of the EGFR, was used.

Expression of the EGFR gene in the xenografts was measured in a variety of ways, including immunocytochemistry, Scatchard analysis of EGF binding, affinity reactions, Western immunoblots, and immunoprecipitations. These analyses led to the following conclusions. (a) Measurable levels of EGFR expression (as assessed by either biochemical or immunological methods) were only present in tumors with gene amplification. (b) The number of EGFR receptors, as assessed by the total binding of EGF to tumor cell membranes, varied widely among tumors with EGFR amplification, although this binding was always at least 5-fold higher than in tumors without EGFR amplification. (c) The affinity of the receptors varied only 5-fold in tumors exhibiting any binding, suggesting that alterations of the gene, when present, did not drastically affect the EGFR domains responsible for the affinity of the EGF ligand for its receptor.

Previous studies have documented rearrangements of the EGFR gene in gliomas (1, 6), but the effect of these rearrangements on protein structure was not established. In the present study, 5 of the 6 xenografts with EGFR amplification had alterations of EGFR gene structure as assessed by Southern blotting, and each was associated with a low molecular weight receptor protein. These proteins were smaller than the normal sized 170-kDa polypeptide which was present in A431 and D-320 MG. Interestingly, the one tumor without EGFR gene alteration (D-320 MG) had a 5-fold higher level of EGFR binding than seen in the tumors with EGFR gene alterations. These small EGFR proteins may be due to genetic alterations in the polypeptide backbone, since they were always associated with gene rearrangements and since they were demonstrated in both immunoprecipitation experiments with monoclonal antibody Ab-1 (528) reactive against the external domain and by Western blot analysis using antibody 2913, reactive against the EGFR intracytoplasmic domain. Alternatively, the size differ-
ence may be due to aberrant glycosylation and/or prototolysis during isolation, but these explanations seem less likely for the following reasons. First, digestion experiments with endoglycosidase F have shown a parallel decrease in the apparent mass of 22P-EGFR from A431 and several of the glioma xenografts, which argues against relative underglycosylation of glioma EGFR as the basis for the size difference (data not shown). Moreover, the use of protease inhibitors which usually protect against receptor fragmentation and the presence of the intact 170-kDa form in identically handled xenografts A431 and D-320 MG argue against proteolytic degradation. Finally, when xenografts D-320 MG and D-270 MG were mixed prior to solubilization, EGFRs of 170 kDa and 145 kDa were still observed, suggesting that proteases present in D-270 MG xenografts were unlikely to completely degrade normal-size 170-kDa EGFR protein originally present in xenograft tissue. However, it is still not possible to entirely exclude prototolysis as the basis for the presence of the low-molecular-weight EGFRs detected in gliomas D-256, D-270, D-298, and D-317; for example, long autoradiographic exposures of the gel in Fig. 5A revealed a faint 170-kDa band in D-256.

Glioma D-245 was unique in that, besides exhibiting a drastically smaller protein product, it displayed no reactivity with either monoclonal antibody Ab-1 (52B) or a polyclonal antibody (451) to the extracellular domain and did not bind EGF. D-245 MG EGFR did, however, bind to an antibody specific for the intracellular portion of the EGFR (2913) and was capable of autophosphorylation (Fig. 5B). These data, as well as the Southern blot experiments presented in Fig. 1, suggest that D-245 MG EGFR had undergone a deletion of a significant portion of the extracellular amino terminal domain, similar to that found in the retroviral v-erbB gene product. As the D-245 MG EGFR is structurally similar to the v-erbB protein, they both might function similarly in conferring a growth advantage; that is, deletion of the EGFR-binding domain in these proteins may result in a constitutively activated state which allows for uncontrolled growth and proliferation (34, 35). A more thorough understanding of the EGFR variants discovered in these gliomas will require cDNA cloning and sequencing and large scale affinity purification of the proteins. The availability of the xenografts described in this work will make such studies feasible.

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Amplification and Expression of the Epidermal Growth Factor Receptor Gene in Human Glioma Xenografts

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