Characterization of the Epidermal Growth Factor Receptor in Human Glioma Cell Lines and Xenografts

Sandra H. Bigner, Peter A. Humphrey, Albert J. Wong, Bert Vogelstein, Joachim Mark, Henry S. Friedman, and Darell D. Bigner

ABSTRACT

Both permanent cultured cell lines and athymic mouse xenografts were established from two human glioblastomas. Biopsies from D-245 MG and D-270 MG contained amplified and rearranged epidermal growth factor receptor (EGFR) genes. Although the gene amplification and rearrangement seen originally was maintained in the xenografts, cultured cell lines established from these biopsies lost the amplified rearranged genes in vitro. Analysis of these cell lines and 11 additional permanent human glioma cell lines with normal EGFR gene copy number showed from 2.7 x 10^5 to 4.1 x 10^5 high affinity EGFRs/cell by radioreceptor assay. The RNase A protection assay showed minimal differences in the quantity of EGFR mRNA among the 13 glioma lines, while the D-245 MG and D-270 MG xenografts expressed approximately 10-20 times as much EGFR mRNA as the corresponding cell lines. Immunoprecipitation of EGFR from these lines, including D-245 MG and D-270 MG, demonstrated only the intact M, 170,000 Da form, while truncated M, 145,000 Da and 100,000 Da EGFR proteins were immunoprecipitated from the D-270 MG and D-245 MG xenografts, respectively. These studies demonstrate that gliomas with amplification of the EGFR gene are capable of establishing in culture but that the amplified rearranged genes are not maintained. Possible explanations are that the abnormal genes are lost during serial passage or that the cells with amplified rearranged genes only represent a minor subpopulation of cells, which are unable to grow in culture. In either case, these observations suggest that high expression and structural abnormalities of EGFR proteins generated by amplification and rearrangement of the EGFR gene provide a growth advantage for gliomas in vivo but not in vitro.

INTRODUCTION

The EGFR gene is amplified in 30-40% of malignant human gliomas (1, 2). Many of these tumors show rearrangement of the amplified genes and these abnormal genes are maintained through propagation in athymic mice (3). The products of the amplified rearranged genes are usually smaller than the normal M, 170,000 Da EGFR, due to deletions involving the region which codes for the extracellular, EGF binding region of the protein (3-5).

Permanent cultured cell lines derived from malignant human gliomas express the EGFR. In contrast to glioma biopsies and xenografts, however, there have been reports of only two lines with amplified EGFR genes, and the level of amplification has been modest (6, 7). The two abnormal EGFR proteins which have been described in vitro are also different from those seen in vivo, in that one was an abnormally large M, 190,000 Da protein and the other one lacked low affinity receptors and failed to respond to EGF (7, 8). These findings suggest that, for gliomas, EGFR genes function differently in in vitro and in vivo environments.

Here, EGFR genes, transcripts, and proteins are characterized for two human glioma cell lines, D-245 MG and D-270 MG, and are compared with xenografts derived from the same glioma biopsies. Both xenografts contain amplified and rearranged EGFR genes which produce abnormally small EGFR proteins. The EGFR genes are not amplified or rearranged in the corresponding cell lines and the EGFR proteins which they express are normal in size and function. These observations demonstrate that xenografts from gliomas with amplified rearranged EGFR genes are appropriate models for studying the abnormal protein products of these genes, while the relevance of EGFR gene and protein function in cultured glioma cells remains to be defined.

MATERIALS AND METHODS

Cell Lines and Xenografts. D-245 MG and D-270 MG cell lines and xenografts were established from glioblastomas from a 70- and a 42-year-old man, respectively. Characterization of the D-245 MG cell line and both xenografts have been reported previously (3, 9). For comparison, cell lines including the A431 squamous cell carcinoma cell line obtained from Dr. Christa Stoscheck (Vanderbilt University) and 11 established malignant human glioma-derived cell lines were used. Lines with the prefix "U" were established at the Wallenberg laboratory in Uppsala by Ponten and Westermark and lines with the prefix "D" were established at Duke University Medical Center. All lines except D-336 MG have been characterized previously (9-11). D-336 MG was established from a gliosarcoma from a 68-year-old man. The karyotype of this original tumor has been published (12).

Cultures are routinely carried in Richter's (13) improved minimal essential medium (Grand Island Biological Co., Grand Island, NY), supplemented with 10% heat-inactivated fetal calf serum, 10 mm N-2-hydroxyethyl piperazine N'-2-ethanesulfonic acid buffer, and 584 mg glutamine/liter, and are incubated at 37°C in a 5% CO2 atmosphere. The cell lines are tested every 10th passage for the presence of Mycoplasma contamination by testing the ability of the conditioned medium to convert deoxyadenosine to adenine (in the presence of an inhibitor of adenosine deaminase) and thymidine to thymine (14). Upon receipt in this laboratory, D-343 MG, C2I2, U-373 MG, U-118 MG, and U-251 MG were contaminated with Mycoplasma. These lines were treated with 10 μg/ml chlorotetracycline daily and increasing periods of heating at 42°C for 2 months. Following treatment, they were grown in antibiotic-free medium for 4 weeks, retested, and found to be free of Mycoplasma. The other cell lines have been consistently negative for Mycoplasma contamination.

Chromosomal Analysis. The identity of each cell line and xenograft was confirmed by karyotype prior to the initiation of these studies. Xenografts were dissociated by incubation for 12-24 h in collagenase (8 mg/ml) in zinc ion solution medium at 37°C. Cultured cells were passaged and cultured until they achieved exponential growth. Cells in both types of preparations were arrested in mitosis by treatment with colcemid, and chromosomal spreads were prepared and G-banded as described previously (15).

Tumorigenicity Testing. For D-270 MG, U-343 MG, and D-
336 MG, cells were grown in culture, resuspended in serum-free zinc option medium, and inoculated s.c. into 1-3 athymic mice/cell line at 1 × 10^6 cells/mouse. Tumorigenicity testing for the other 10 lines has been reported previously (9, 10).

Analysis for Gene Amplification by Hybridization. DNA was purified from cultured A431 cells and the 13 glioma lines and from frozen xenograft tissue derived from D-245 MG and D-270 MG, using methods detailed previously (11). Five to 7 µg of DNA were cleaved with EcoRI, separated by electrophoresis through a 1% agarose gel, blotted onto a nylon membrane, and hybridized with the EGF probe labeled with [32P]-dCTP via the random primer method. Washing of filters and autoradiography were as described (16). The EGF probe used was the 1.6-kilobase EcoRI fragment of pE7, a complementary DNA clone of EGF mRNA generously provided by Drs. G. Merlino and I. Pastan (NIH) (17). The signals were removed by boiling, and the filters were rehybridized with a 1.0-kilobase EcoRI/BamHI fragment of pNB containing part of the second exon of the N-myc gene (18).

Ribonuclease Protection. Total RNA was isolated by the acid-guanidium extraction method described by Chomczynski and Sacchi (19). The integrity of RNA was assessed by denaturing gel electrophoresis and staining with ethidium bromide. 32P-labeled RNA transcripts were synthesized from 2.5 µg of plasmid DNA and [α-32P]-UTP to a specific activity of 1 × 10^8 cpm/µg using T7 polymerase, as described (20). The RNA transcripts were hybridized to 20 µg of total RNA from cultured A431 cells and the 13 glioma lines and from frozen xenograft (Vanderbilt University Medical Center, Nashville, TN 37232) samples. Stringent washing conditions were used for hybridization and the hybridized RNA was subjected to electrophoresis through 6% polyacrylamide gels. Autoradiography was performed by exposing Kodak AR-5 film for varying lengths of time (1-12 h) at −70°C.

RESULTS

Chromosomal Analysis. Cells of D-245 MG xenograft in passage 28, D-245 MG cell line in passage 87, and D-270 MG xenograft in passage 10 had stemlines similar to those reported previously (3, 9). DMs were present in virtually all cells of both xenografts but could not be found in the D-245 MG cell line. Cells of the D-270 MG cell line were 47,XY,i(6p)(t(9q13q)),+ring,+IDMs (Fig. 1). None of the cell lines or xenografts contained homogeneously staining regions. Karyotypes of A431 and 6 of the remaining 11 glioma cell lines contained the same stemline karyotypes as had been reported previously (9, 11, 15, 26, 27). The five lines which have not had stemline karyotypes published previously are as follows. D-37 MG: S=96-97, XX, -X, -X, -10, +11, +19, +20, -22, -22, +del(1q)(11), +i(2q), del(7q)- (q21), +del(7q)(q21), t(9q22q), +t(9q22q), +der(3)(t(3;p11);?), der(13)(t(13q?))(q32–34?); der(21)(t(21q?);q22?), +mar, +frag (expressed as deviations from tetraploid). D-336 MG: S=74, XXXY, -Y, -2, -4, -5, -7, 9, -9, -10, -11, -12, -13, -14, -14-16, -16, -18, -19, -22, -22, del(3q)(q25), del(11p11) (p11), del(13)(q11q22), der(12)(9q13)(11q11p11) (expressed as deviations from tetraploid). U-373 MG: S=121-122, XXXXY, -4, +5, -10, -11, -13, +14, +14, -15, -18, -18, -19, +20, +22, -i(3q), +i(3q), del(6q)(q16), del(6q)(q16), del(6q)(t(13q21), +del(6q)(q13q21), +del(6q)(q13q21), inv(9)(p11q13), inv(9)-p11q13), del(12) (q15q23), +del(12)(15q25), +der(1)(17q)- (q31?); +der(6)(6q?); (q23?); +der(16)(t(16p11q6), +del(16q)- (11p16q), +der(16)(11p16q), +der(16)(11p16q), +DMS (expressed as deviations from pentaploid). U-410 MG: S=49, XY, +7, +7, -10, del(19)(cen), +der(1)(1q10q), +der(19)(13q19); U-119 MG: S=96-98, XXXY, -Y, -4, -5, +11, +12-16, -18, -19, +20, +20, +22, +i(1q), +der(3)(13q33), del(6q)- (13q33), +del(6q) (q15), +del(6q)(p23q11), +del(6q)(p23q11), del(9)(p21), +del(9)(p21), del(10)(p13), del(10)(p13), +der(3)(3q15q)+1-5mars (expressed as deviations from tetraploid).

Tumorigenicity. Cells from U-343 MGAci2:6 grew progressively s.c. in 3 of 3 mice and could be serially transplanted into new generations of animals. Mice bearing D-270 MG and D-336 MG cells were observed for 1 year, but no tumors grew. Results of tumorigenicity testing for the other 10 lines have been reported previously, as follows. U-373 MG, U-118 MG, D-54 MG, D-263 MG, D-247 MG, and U-251 MG grew...
progressively when inoculated s.c. into athymic mice (9, 10). D-37 MG, D-245 MG, and D-32/MGac12:6MG did not. U-410 MG grew initially but regressed (10).

Gene Number and Structure. Hybridization of DNA samples derived from the 13 glioma cell lines with the EGFR and N-myc probes showed only normal gene copy numbers. In particular, DNA from cell lines D-270 MG and D-245 MG showed no evidence for EGFR gene amplification or rearrangement, while xenografts derived from the same original tumors showed approximately 10–25-fold amplification of the EGFR gene and retained the same pattern of gene rearrangement as had been demonstrated in the biopsies from which they had been derived (Fig. 2).

Quantitation of EGFR mRNA. The RNase A protection assay (Table 1, Fig. 3) demonstrated that all of the cultured glioma cell lines varied by no more than 3-fold in EGFR mRNA content. In contrast, the D-270 MG and D-245 MG xenografts contained 8–10 times and >20 times as much EGFR mRNA, respectively, as did the corresponding cultured cell lines and also contained more mRNA than xenograft D-263 MG, which did not have EGFR gene amplification.

Quantitation of Glioma Cell Line EGFR Protein Expression by Scatchard Analysis of 125I-EGF Binding. Saturation curves of the direct binding of increasing amounts of 125I-EGF to cultured glioma cells are shown in Fig. 4. The Scatchard plots of these data were usually curvilinear (Fig. 5); the affinity constants and the number of EGFRs per cell for both the low and high affinity receptor classes are presented in Table 2. The glioma cell lines expressed a highly variable number of receptors, from 0.27 × 10^6 EGFRs/cell (U-251 MG) to 1.6 × 10^6 EGFRs/cell (D-37 MG). In comparison, the A431 squamous cell carcinoma line, which is known to express very high levels of EGFR, had 4.0 × 10^6 low affinity EGFRs/cell. The association constant of the EGF-glioma EGFR binding reaction also varied over wide range, from 2.5 × 10^7 M⁻¹ to 5.6 × 10^10 M⁻¹.

Apparent Molecular Weight of Glioma EGFR. Fig. 6 exhibits the relative sizes of 32P-EGFR immunoprecipitated from cultured glioma cells with monoclonal antibody Ab-1 (528) to the extracellular EGFR domain and with a polyclonal antibody reactive against the intracytoplasmic portion of EGFR (Ab-2913). The normal-sized M, 170,000 Da EGFR was observed in all six glioma cell lines (Fig. 6, lanes 2–7 and 9–14) and the A431 squamous cell carcinoma line (Fig. 6, lanes 1 and 8); a M, 150,000 Da proteolytic product was present in glioma U-343 MGac12:6 (Fig. 6, lanes 3 and 10). The M, 170,000 Da bands in gliomas D-245 MG and D-37 MG are difficult to visualize at this autoradiographic exposure time (of 0.5 h) but were readily identified in longer exposures. Five additional glioma cell lines (U-373 MG, D-45 MG, U-410 MG, U-118 MG, and U-251 MG) expressed a normal-sized EGFR with...
intrinsic kinase activity (data not shown). D-54 MG also expressed the previously described M, 190,000 Da variant (8). The extent of $^{32}$P incorporation into the glioma EGFR molecule, an indicator of the intrinsic EGFR tyrosine kinase activity, did not correlate with receptor number. Some cell lines with the higher receptor numbers (A431, U-343 MGaC12:6, and D-247 MG) exhibited a more intense EGFR band than cell lines with lower receptor numbers (D-336 MG, D-270 MG, and D-245 MG), but other lines, such as D-37 MG, which also had large numbers of EGFR did not autophosphorylate well (Fig. 6, lanes 2 and 9).

Fig. 7 presents a comparative electrophoretic pattern of $^{32}$P-

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Relative mRNA level</th>
<th>Xenografts</th>
<th>Relative mRNA level</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-37 MG</td>
<td>2-3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U-343 MGaC12:6</td>
<td>3-4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-247 MG</td>
<td>~1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-336 MG</td>
<td>~1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-245 MG</td>
<td>&gt;1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U-325 MG</td>
<td>~1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-32 Cl5 MG</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1 EGFR mRNA in glioma cell lines and xenografts as determined by RNase A protection

- Total cellular RNA was hybridized with an 883-base antisense probe from the EGFR, cleaved with RNase A, and electrophoresed on a 6% acrylamide-urea gel. Relative amounts of EGFR mRNA were determined on autoradiograms by comparison of glioma cell lines and xenografts with the signal from D-32 Cl5 MG, which served as the standard and was considered to be 1.

- Parameters were determined by Scatchard analysis of binding data presented in Fig. 4.

- Estimated affinity and number of EGF receptors on human glioma cell lines

<table>
<thead>
<tr>
<th>Receptor class</th>
<th>High affinity</th>
<th>Low affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell line</td>
<td>$K_a$ (m$^{-1}$)</td>
<td>Receptors/cell</td>
</tr>
<tr>
<td>A431*</td>
<td>$1.5 \times 10^9$</td>
<td>$1.3 \times 10^9$</td>
</tr>
<tr>
<td>D-37 MG</td>
<td>$1.9 \times 10^9$</td>
<td>$4.1 \times 10^9$</td>
</tr>
<tr>
<td>U-343 MGaC12:6</td>
<td>$3.0 \times 10^9$</td>
<td>$2.5 \times 10^9$</td>
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<tr>
<td>D-247 MG</td>
<td>$1.9 \times 10^9$</td>
<td>$2.7 \times 10^9$</td>
</tr>
<tr>
<td>D-336 MG</td>
<td>$7.7 \times 10^9$</td>
<td>$7.2 \times 10^9$</td>
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<tr>
<td>D-270 MG</td>
<td>$1.4 \times 10^9$</td>
<td>$3.4 \times 10^9$</td>
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<tr>
<td>U-373 MG</td>
<td>$2.6 \times 10^9$</td>
<td>$1.2 \times 10^9$</td>
</tr>
<tr>
<td>D-54 MG*</td>
<td>$7.7 \times 10^9$</td>
<td>$7.2 \times 10^9$</td>
</tr>
<tr>
<td>D-247 MG*</td>
<td>$2.5 \times 10^9$</td>
<td>$1.1 \times 10^9$</td>
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<tr>
<td>D-263 MG</td>
<td>$5.5 \times 10^9$</td>
<td>$4.1 \times 10^9$</td>
</tr>
<tr>
<td>D-245 MG</td>
<td>$5.3 \times 10^9$</td>
<td>$1.8 \times 10^9$</td>
</tr>
<tr>
<td>U-325 MG</td>
<td>$3.3 \times 10^9$</td>
<td>$0.27 \times 10^9$</td>
</tr>
<tr>
<td>D-32 Cl5 MG*</td>
<td>$2.9 \times 10^9$</td>
<td>$1.0 \times 10^9$</td>
</tr>
</tbody>
</table>

* A431 squamous cell carcinoma line.

* Curvilinear Scatchard plots not obtained, so straight line fit was used to determine the affinity and receptor number for the one class of EGFR.
EPIDERMAL GROWTH FACTOR RECEPTOR IN GLIOMAS

EGFR immunoprecipitated from glioma xenograft tissue and glioma cultured cells, which were derived from the same biopsy tissue. Monoclonal antibody Ab-1 (528) immunoprecipitated the intact \( M, 170,000 \) Da EGFR from both A431 xenograft tissue and A431 cell culture (Fig. 7, lanes 1 and 2). The normal-sized receptor was also immunoprecipitated from glioma cell lines and D-270 MG (Fig. 7, lane 3) and D-245 MG (Fig. 7, lane 4). In contrast, glioma xenografts D-270 MG and D-245 MG expressed a slightly smaller EGFR (D-270 MG) (Fig. 7, lane 4) and an EGFR which is not immunoprecipitated by the monoclonal antibody Ab-1 (528) reactive against the external domain (D-245 MG) (Fig. 7, lane 6). D-245 MG EGFR is immunoprecipitable by the antibody 2913 reactive against the intracytoplasmic portion of EGFR and is markedly smaller (with a doublet at \( M, 80,000 \) Da and 100,000 Da) (Fig. 7, lane 10) than the normal-sized \( M, 170,000 \) Da EGFR that is expressed by D-245 MG cell culture (Fig. 7, lane 9). The normal-sized \( M, 170,000 \) Da D-245 MG cell culture EGFR band in Fig. 7, lane 9, was more easily visualized at longer autoradiographic exposures and could be seen in Fig. 7, lane 5.

DISCUSSION

Permanent cultured cell lines derived from human neoplasms provide useful in vitro models for defining a variety of biological properties of these neoplastic cells. Gene amplification, in particular, has been studied in experimental settings in vitro, since this property can be maintained only under conditions of selective pressure.

The human tumor type which is best characterized for gene amplification both in vivo and in vitro is human neuroblastoma. While approximately 38% of resected neuroblastoma contain amplification of the \( N-myc \) gene, amplification of this gene characterizes virtually all permanent cultured neuroblastoma cell lines, suggesting that it is necessary for these cells to establish in culture (18, 28). For malignant gliomas, in contrast, 30–40% of biopsies contain amplification of the EGFR gene (1, 2). Among the more than 50 glioma cell lines which have been analyzed to date, however, there are only two examples of EGFR gene amplification, and both lines show only a modest increase in gene copy number (6, 7). There are several theoretical explanations for this observation. The demonstration that D-245 MG and D-270 MG, two gliomas with amplification of the EGFR gene, established in culture excludes the possibility that gliomas with amplification of this gene are not capable of establishing in culture and negates the possibility that gliomas with EGFR gene amplification have not been tested in culture for their ability to establish. For these two tumors, amplification and rearrangement of the EGFR gene, which was demonstrated in initial biopsies and xenografts, was lost as these tumors established in vitro. This observation suggests that the selective conditions in vivo which favor amplification of this gene are not present in culture. The inability of the D-245 MG and D-270 MG cell lines to grow when injected into athymic mice supports this concept. Furthermore, studies on NIH 3T3 cells show that transfection and overexpression of the EGFR can endow this cell line with the ability to grow in athymic mice (29).

The cultured lines D-270 MG and D-245 MG contain the number of EGFR gene copies appropriate for their copy number of chromosome 7, fail to show any rearrangement of this gene, and express appropriate amounts of structurally normal EGFR mRNA and protein for the number of gene copies which they contain. In this respect, they resemble the other glioma cell lines described here, which include three lines for which the initial biopsies were available and which did not contain EGFR gene amplification in the original human tumors.

Both D-245 MG and D-270 MG biopsies and xenografts contained rearranged as well as amplified EGFR genes, and these tumors bound EGF with a lesser capacity than tumors with amplified, structurally normal EGFR genes (3). This observation suggests that these abnormal proteins may be capable of functioning in the absence of EGF and, therefore, cells expressing these abnormal EGFRs may possess a growth advantage over cells expressing only the normal EGFR in the presence of low levels of EGF. It is not possible to determine the amounts of EGF accessible to glioma cells in vivo and in xenografts, and no exogenous EGF was added to the culture.
medium used to generate and propagate these cell lines. Nevertheless, since transforming growth factor α mRNA is frequently expressed by glioma cell lines in culture (11), the possibility remains that higher levels of EGFR ligands may be present in vitro than in vivo and thus may remove the selective advantage imparted by the amplified rearranged EGFR genes.

Many glioma cell lines express platelet-derived growth factor and/or its receptor (11). Platelet-derived growth factor as well as other growth factors and hormones are present in fetal calf serum, raising the alternative possibility that differences in the levels of these substances between the in vitro and in vivo settings may be the selective pressure producing the loss or maintenance of EGFR gene amplification and rearrangement in these settings, respectively. Attempts to establish cultured cell lines from the D-270 MG and D-245 MG xenografts in defined media containing various growth factors may allow maintenance of the amplified and rearranged genes and may permit precise identification of the factors which interact with the EGFR in gliomas.

The observation that the amplified and rearranged EGFR genes were lost as D-245 MG and D-270 MG established in culture but were maintained in xenografts is in contrast to examples of squamous cell carcinomas such as A431 cells, where amplification of the EGFR gene appears to provide a growth advantage in vitro (30). One explanation for this difference is that the EGFR genes and proteins may function differently in glioma cells than in squamous cell carcinoma cells. Alternatively, the difference may be due to the nature of the rearrangements of the amplified EGFR genes in these two glioma cell lines.

The high incidence of amplification and rearrangement of the EGFR gene in human gliomas is an interesting and undoubtedly biologically significant property of these tumors. Further analysis of the glioma cell lines and xenografts described here may provide insight into the way in which these amplified genes function in the in vivo and in vitro settings.

REFERENCES

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