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 × 10^6 to 4.1 × 10^6 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 Mycoplasma.

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; DMs, double-minute chromosomes; SDS, sodium dodecyl sulfate.
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 (16). 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 EGFR probe labeled with 32P-dCTP via the random primer method. Washing of filters and autoradiography were as described (16). The EGFR probe used was the 1.6-kilobase EcoRI fragment of pE7, a complementary DNA clone of EGFR 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 generated in vitro from the 0.7-kilobase EcoRI fragment of pE7 cloned into the plasmid Bluescript using T3 or T7 RNA polymerase. Ribonuclease protection was performed as described by Winter et al. (20) with the following modifications: hybridizations were performed in a final volume of 10 μl, only RNase A at 12.5 μg/ml was used, and the RNase A and proteinase K digestions were performed at room temperature for 30 min.

Binding of 125I-EGF to Glioma Cell Lines. Cells were plated in 24-well plates, at 1.0 × 10^5 cells/well in 1 ml of zinc option medium containing 10% fetal calf serum. Incubation was for 18–24 h at 37°C. The wells were washed 3 times with 1 ml of binding buffer (20 mM N-2-hydroxyethyl piperazine N’-2-ethanesulfonic acid, 0.1% bovine serum albumin, pH 7.4), and 125I-EGF (Amersham) in binding buffer was added over a concentration range of 0–25 nM. Each concentration point was assayed in quadruplicate. Nonspecific binding was monitored by adding a 100-fold molar excess of unlabeled EGF (Collaborative Research) to four wells with the highest concentration of 125I-EGF. After a binding reaction of 1 h at room temperature, unbound 125I-EGF was aspirated from the plated glioma cells and the wells were washed 3 times with binding buffer. The cells were removed from the wells by the addition of 1 N NaOH for at least 1 h at 37°C, followed by transfer to tubes, which were counted in a Packard Auto Gamma spectrometer. The binding data were analyzed by the Scatchard method on an IBM PC/AT computer using the software program EBDA (equilibrium binding data analysis), written by G. A. McPherson (21,22) and published previously (21). 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).

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(9q13 q)),+ring,DMs (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 those had been reported previously (9,11,15,26,27). The five lines which have not had karyotypes published previously are as follows. D-37 MG: S=96–97, XX, –X, –X, –10, +11, +19, +20, –22, –22, +del(1)(q11), +i(2q), del(7)(q21), +del(7)(q21), +del(7)(q22), +del(9)(3pter,p11), +der(13)(q13?), +der(13)(q32–34?), +del(21)(q21, q22, +mar, +frag (expressed as deviations from tetraploid). D-336 MG: S=74, XXXY, –Y, –X, –4, –5, –7, –9, –9, –10, –11, –12, +13, –14, –14, –16, –16, –18, –19, –22, +del(3)(q25), +del(11)(p11), +del(13)(q11q22), +del(12)(9q12)(p11q11) (expressed as deviations from tetraploid). U-373 MG: S=122–122, XXXXY, –4, –5, +10, –11, –13, +14, +14, –15, –18, –18, +19, +20, +21, –i(3q), +i(3q), +del(6)(q16), +del(6)(q16), +del(6)(q16), +del(6)(q13q21), +del(6)(q13q21), +inv(9)(p11q13), +inv(9)(p11q13), +del(12)(q15q23), +del(12)(q15q23), +del(17)(q17– q31?), +del(18)(6;7) (q23,?), +del(16)(11p16q), +der(16)t(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)(11q10q), +der(19)(13q19–q14), U-118 MG: S=96–98, XXXY, –Y, –4, –5, –11, +12, 16, –18, –19, +20, +20, +22, +i(1q), +del(3)(q13q33), +del(6)(q13q33), +del(6)(q15q), +del(6)(p23q11), +del(6)(p23q11), +del(9)(p21), +del(10)(p13), +del(10)(p13), +der(3)(3q15q)+1–5mars (expressed as deviations from tetraploid). Tumorigenicity. Cells from U-343 MG cell line 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, U-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/MGCl2:6MG 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 × 10^4 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 MGCl2:6 (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 32P 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 MGA12:6, 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 32P-

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

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Relative mRNA level</th>
<th>Xenografts</th>
<th>Relative mRNA level</th>
</tr>
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<tbody>
<tr>
<td>D-37 MG</td>
<td>2-3</td>
<td>8-10</td>
<td></td>
</tr>
<tr>
<td>U-343 MG</td>
<td>3-4</td>
<td>2.5</td>
<td></td>
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<tr>
<td>D-263 MG</td>
<td>5-6</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>D-245 MG</td>
<td>7-8</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>U-410 MG</td>
<td>9-10</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>U-251 MG</td>
<td>12-13</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td>D-263 CG</td>
<td>15-16</td>
<td>3.7</td>
<td></td>
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</tbody>
</table>

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Fig. 2. Upper, Southern blot analysis of DNA from A431 cells (lane 1), normal human lymphocytes (lane 2), D-270 MG xenograft (lane 3) and cell line (lane 4), and D-245 MG xenograft (lane 5) and cell line (lane 6), cleaved with EcoRI and hybridized with the EGFR probe, demonstrates amplification of this gene in A431 cells and amplification and rearrangement of this gene in DNA from both glioma xenografts. In contrast, only normal copy numbers of unarranged EGFR genes are seen in both glioma cell lines. Lower, the filter shown in the upper panel rehybridized with an N-myc probe demonstrates similar amounts of RNA in each lane.

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Fig. 3. RNase A protection of cell lines and xenografts. 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. The RNAs used were from the cell lines U-410 MG (lane 1), U-373 MG (lane 2), U-343 MGA12:6 (lane 3), U-118 MG (lane 4), U-251 MG (lane 5), D-32 Cl2 MG (lane 6), D-37 MG (lane 7), D-54 MG (lane 8), D-270 MG (lane 9), D-247 MG (lane 10), D-263 MG (lane 11), D-270 MG (lane 12), and D-336 MG (lane 13), the xenograft of D-245 MG (lane 14), and the xenograft of D-270 MG (lane 15).

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Fig. 4. Saturation analysis of 125I-EGF binding to 13 glioma cell lines. Increasing concentrations of 125I-EGF were added to glioma cells cultured in 24-well plates. Unbound 125I-EGF was removed by washing and glioma cells with bound 125I-EGF were harvested with 1 N NaOH. Binding was followed by gamma-spectrometry.
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 normalized 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 normalized \( M, 170,000 \) 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 vivo than in vitro 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 gliomas.

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.

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