Epidermal growth factor receptors on ependymomas and other brain tumors

WALTER A. HALL, M.D., MARSHA J. MERRILL, PH.D., STUART WALBRIDGE, B.S., AND RICHARD J. YOULE, PH.D.

Biochemistry Section, Surgical Neurology Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland

Epidermal growth factor receptor (EGFR) and transferrin receptor levels were determined in 14 intracranial neoplasms (four glioblastomas multiforme, four medulloblastomas, four ependymomas, one cerebellar astrocytoma, and one acoustic neurinoma) and in four samples of "normal" brain tissue. A competitive radioreceptor assay with 125I-epidermal growth factor and 125I-transferrin was performed using the primitive neuroectodermal tumor-derived TE-671 tissue-culture cell line as a standard. Epidermal growth factor receptors were present on TE-671 cells, all four ependymomas, and two of the four glioblastomas multiforme. The number of EGFR's per cell for ependymomas were estimated to range from 1000 to 6000. Transferrin receptors were detected on TE-671 cells, two of the four medulloblastomas, and one of the four glioblastomas multiforme. A cell surface binding assay, performed directly on the rat ependymal cell monolayer, was also analyzed.

The identification of EGFR's on ependymomas and TR's on medulloblastomas suggests that malignant central nervous system tumors that spread by cerebrospinal fluid pathways may be treatable by intrathecal antibody-toxin conjugates. The presence of EGFR's on all of the ependymomas may reflect a role of the receptor in the malignant phenotype of this tumor.

KEY WORDS • epidermal growth factor receptor • transferrin receptor • brain neoplasm • immunotoxin • ependymoma • medulloblastoma

growth factors such as epidermal growth factor (EGF) and transferrin are proteins that stimulate cell proliferation through binding to high-affinity cell surface receptors and affect gene expression and cell division in normal and neoplastic transformed states. Some cancer cells produce their own growth factors and, in an autocrine manner, initiate a state of continuous proliferation. Increased expression of growth factor receptor levels may also enable some tumor cells to respond to growth factors produced by normal tissues.

Epidermal growth factor is a 53 amino acid peptide (molecular weight (M,) 6045) purified first from mouse submaxillary glands and later from human urine. The c-erb B gene product is the EGF receptor (EGFR) and has a 90% amino acid sequence homology with the v-erb B oncogene protein of the erythroblastosis virus. The c-erb B gene bears nucleotide sequence homology to the neu oncogene.

Transferrin (M, approximately 90,000) is a glycoprotein responsible for iron binding and transport in serum. The cell surface glycoprotein P97 (M, 97,000) is present in most human melanomas and is homologous to transferrin; it was found capable of binding to iron and may be functionally related to transferrin and/or to the transferrin receptor (TR). The genes for p97, transferrin, and the TR have all been mapped to chromosome 3 in humans. Proliferating cells express a greater number of TR's than do nondividing cells. Gatter, et al., have shown that some tumors selectively express TR's relative to most normal tissues.

Cell surface receptors for EGF and transferrin have been demonstrated on various human tumor types. Epidermal growth factor receptors have been found on human squamous-cell carcinomas of the lung, breast carcinomas, and glioblastomas multiforme. In vitro studies have also shown EGFR's on a variety of tissue-culture cell lines. Transferrin receptors have been found on human breast carcinoma tissue and on a human melanoma cell line.

Materials and Methods

Tissue Samples

Fresh samples of brain-tumor tissue were obtained in the operating room, immediately placed on dry ice,
and stored at −70°C. The samples collected consisted of four medulloblastomas, four ependymomas, four glioblastomas multiforme, one cerebellar astrocytoma, and one acoustic neurinoma. "Normal" brain samples were taken from the frontal lobe and the cerebellum of two patients within 8 hours after death. These samples were processed in the same manner as the surgical specimens. Histological examination of the autopsy material did not disclose any abnormalities.

For comparison with the binding results from the surgical tissue specimens, a solid human TE-671 primitive neuroectodermal tumor (PNET) was grown from cells in tissue culture* in the flank of an athymic nude mouse (nu/nu genotype, National Institutes of Health). The animal was sacrificed and the tumor was placed on dry ice with storage at −70°C.

**Transferrin Preparation**

Human transferrin† was saturated with iron by the following method. Six mg of transferrin was dissolved in 1 ml of 0.25 M Tris-Cl (pH 8.0)/10 mM NaHCO₃. Next, 20 μl of 100 mM disodium nitrilotriacetate/12.5 mM FeCl₃ was then added to the transferrin solution, which was incubated for 30 minutes at 37°C. This was then passed over a PD-10 column‡ previously equilibrated with 0.15 M NaCl/0.02 M Tris-Cl (pH 7.4). The amount of transferrin-bound iron was estimated from the A₆₆₅₉/cm²: A₈₀₀₉ ratio and found to be 0.045 to 0.051. The protein concentration was determined by the method of Lowry, et al., using bovine serum albumin (BSA) as the standard.

Diferric transferrin (1.25 μg/25 μl) in 50 μl 0.15 M NaCl/0.02 M Tris-Cl (pH 7.4) was combined with 50 μl of a lactoperoxidase/glucose oxidase solid-phase reagent suspension.§ Ten μl of Na¹²⁵I (556 mCi/ml) and 25 μl of 1% beta-D-glucose were added to the sample. The reagents were incubated for 30 minutes at room temperature. The reaction was stopped with 100 μl of 1.0% NaI, in phosphate-buffered saline (PBS: 10 mM NaH₂PO₄, 150 mM NaCl, pH 7.4)/0.2% BSA. The mixture was immediately passed over a PD-10 column equilibrated with PBS/0.2% BSA. Fractions were collected.

**Membrane Preparation**

Frozen brain-tumor tissue, "normal" brain tissue, and solid TE-671 were washed twice in PBS. All surface vessels and obvious blood clot were removed. Membranes were prepared as described by Kiess, et al. The tissue was homogenized on ice in four volumes of 50 mM HEPES (pH 7.4), 0.25 M sucrose, 1.25 μg/ml antipain, and 50 μg/ml phenylmethylsulfonyl fluoride using the polytron homogenizer. The homogenate was centrifuged for 10 minutes at 2000 rpm* and the supernatant was saved. The pellet was rehomogenized, centrifuged as above, and the supernatants were combined. The supernatant was brought to a concentration of 100 mM NaCl and 0.2 mM MgSO₄ by the addition of 10 volumes of stock solution and then centrifuged in the ultracentrifuge† at 37,500 rpm for 90 minutes.

For the EGF radioreceptor assay, the supernatant was discarded and the pellet was resuspended for 30 minutes in 0.1 M sodium acetate buffer (pH 5.0). It has been shown that EGF dissociates from its receptor at pH 5.0. The suspension was centrifuged in a microfuge for 10 minutes. For both receptor assays, the supernatant was discarded and the pellet was resuspended in 50 mM Tris-HCl (pH 7.4), 1.25 μg/ml antipain, and 50 μg/ml phenylmethylsulfonyl fluoride. Protein content was determined by the method of Lowry, et al. The membrane preparation was stored at −70°C.

**Binding Assays**

A competitive radioreceptor binding assay was performed on the TE-671 membrane preparation using ¹²⁵I-EGF‡ and ¹²⁵I-transferrin, prepared as above. A protocol was used that demonstrated TR saturation by 4 hours at 4°C. The assay was performed in Eppendorf microfuge tubes where the final volume was 400 μl. The components of the assay included: 1) 100 μl binding buffer, RPMI 1640 medium, 10 mM HEPES (pH 7.4), and 0.2% BSA; 2) a 100-μg membrane preparation in 100 μl binding buffer; 3) 80,000 to 90,000 cpm ¹²⁵I-EGF or ¹²⁵I-transferrin in 100 μl binding buffer; and 4) 100 μl of EGF (0 to 2.3 nM) or ¹²⁵I-transferrin (0 to 200 nM). Ten dilutions of both cold ligands were incubated at 4°C for 4 hours. The reaction was stopped by centrifugation in the microfuge for 5 minutes. The supernatant was aspirated and the tubes were drained by inversion. The tube tips containing the pellet were cut off and the radioactivity was counted in the Beckman gamma counter. A Scatchard analysis was performed and the affinity constants of the EGFR and the TR were determined. The number of receptors/μg membrane protein was calculated.

Because of a limited amount of available tissue, duplicate two-point radioreceptor binding assays were performed on brain tumor and samples of "normal" brain tissue using an absence or presence of cold ligand (2.3 nM EGF and 200 nM transferrin). The compo

* Cells obtained from American Type Culture Collection, Rockville, Maryland.
† Human transferrin obtained from Sigma Chemical Company, St. Louis, Missouri.
‡ Sephadex G-25 manufactured by Pharmacia, Uppsala, Sweden.
§ Enzymo beads manufactured by Bio-Rad, Richmond, California.
// Polytron homogenizer manufactured by Brinkmann Instruments, Westbury, New York.
* Centrifuge, Model TJ-6, manufactured by Beckman Instruments, Irvine, California.
† Ultracentrifuge, Model L8-70, manufactured by Beckman Instruments, Irvine, California.
‡ ¹²⁵I-EGF obtained from New England Nuclear, Boston, Massachusetts.
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components of the binding assay were as described above. At the time the assay was performed on the tumor or "normal" brain membrane preparations, a similar assay was also performed on solid TE-671 tissue as a positive control. The percent specific binding (specific cpm bound/total cpm added × 100)/100 µg seen in the brain-tumor samples was compared to that seen on solid TE-671 tissue, and a comparative binding percentage was calculated. From this percentage, we were able to estimate the number of receptors/µg membrane protein of the brain-tumor tissue samples based on the number of receptors present on TE-671 cells.

Cell Number Determination

The TE-671 cells were grown in tissue culture flasks in Dulbecco's minimal essential medium (DMEM) containing 10% fetal calf serum, 2 mM glutamine, 1 mM sodium pyruvate, 10 µg/ml gentamicin, 10 mM HEPES (pH 7.2), nonessential amino acids, and sodium bicarbonate. Cultured cells were washed twice in PBS and incubated at 37°C for 15 minutes in 10 mM ethylenediaminetetra-acetic acid in PBS (pH 7.4). The cells were harvested using a rubber policeman and centrifuged twice at 1000 rpm for 5 minutes and the medium was decanted. The pellet was homogenized as outlined in the above section and the membranes were prepared. The amount of protein in the membranes was determined by the method of Lowry, et al. After determining the number of TE-671 cells that yield 1 µg membrane protein, we were able to estimate the number of binding sites/cell.

Ependymal Surface Binding Assay

Sprague-Dawley rats, each weighing 200 to 300 gm, were anesthetized and decapitated, and their brains were removed. The hemispheres of each brain were separated and the lateral ventricle was unfolded to provide a uniform ependymal surface. The ventricular surface was washed with 0.1 M sodium acetate buffer (pH 5.0) to remove any bound EGF originating from the cerebrospinal fluid (CSF). A 1 cm-long cylindrical well, fashioned from a 1-ml polyethylene microtest tube, was mounted with agarose in Gelfoam on the ependymal surface so that the entire internal diameter of the bottom of the well was in contact with the ependyma. The brains were immersed in DMEM with 2 mM glutamine and gentamicin (10 µg/ml) Petri dishes at 4°C. Care was taken not to fill the well with the medium from the dish.

A competitive radioreceptor binding assay was performed within each well on the ependymal cell mono-layer using 125I-EGF and 125I-transferrin. The brains were divided into two groups with three specimens in each group. In one group, 80,000 to 90,000 cpm of 125I-EGF or 125I-transferrin in 100 µl of binding buffer was added to each well. In the second group, 80,000 to 90,000 cpm of 125I-EGF or 125I-transferrin in 50 µl of binding buffer was added to 50 µl of 6.25 × 10^2 nM EGF or 50 µl of 3.25 × 10^4 nM transferrin, respectively, in each well. The assay was incubated at 4°C for 4 hours.

After incubation, the wells were aspirated and removed from the ependymal surface. The ependymal surface in contact with the radiolabeled ligands was placed in a test tube and counted in the gamma counter.

Results

The amount of tissue obtained from the flank-grown solid TE-671 tumor enabled us to perform a complete Scatchard analysis using concentrations of ligand ranging from 3.4 × 10⁻³ nM to 2.3 nM for the EGFR's (data not shown) and 5.2 × 10⁻⁴ nM to 200 nM for the TR's (Fig. 1). From the Scatchard analysis data, we determined the affinity constant (K_a) of the EGFR to be 7.5 × 10⁸ M⁻¹ and that of the TR to be 2.6 × 10⁶ M⁻¹. These affinity constants, found for TE-671 tumor tissue, were comparable to previously reported values for a variety of other tumors. The number of receptors/µg of TE-671 membrane protein was determined from Scatchard analysis to be 2.1 × 10⁷ receptors/µg for EGFR's and 1.8 × 10⁸ receptors/µg for the TR's.

The number of TE-671 cells/µg membrane protein was determined by homogenizing a tissue-culture cell pellet: 8.94 × 10⁶ cells yielded 1 µg of membrane protein. Knowledge of the number of cells/µg membrane protein and the number of receptor sites/cell enabled us to estimate the number of receptor sites/cell. Because

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$^\text{§}$ Sprague-Dawley rats obtained from Taconic Farms, Germantown, New York.
of the limited amount of human tumor tissue available to us, we could not do a complete Scatchard analysis on most specimens. We therefore utilized a two-point binding assay on frozen brain-tumor samples using solid TE-671 tissue as a reference. If we assumed that the "normal" brain tissue and tumor tissue have the same affinity for EGF and transferrin as TE-671 solid tissue, this two-point assay allows us to estimate the number of receptors/µg membrane protein and therefore to estimate roughly the number of binding sites per cell (Tables 1 and 2). We could not detect EGFR or TR on the "normal" brain samples. The level of sensitivity of the assay could detect approximately 1 × 10^3 binding sites/cell for both receptor types.

Epidermal growth factor receptor levels were detectable on TE-671, two of four glioblastomas multiforme, and all four ependymoma samples. The cerebellar astrocytoma and the acoustic neurinoma were without detectable EGFR’s. Table 1 demonstrates the percent specific binding/100 µg membrane protein based on TE-671 for the EGFR’s. Transferrin receptors were detected on TE-671 and on two of four medulloblastoma samples. One of four glioblastomas multiforme expressed significant numbers of TR’s. All four ependymomas, one cerebellar astrocytoma, and one acoustic neurinoma did not express detectable levels of TR on the "normal" brain samples. The level of sensitivity of the assay could detect approximately 1 × 10^3 binding sites/cell for both receptor types.

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Since EGFR’s were found on all four ependymoma samples, we wondered if normal ependymal cells expressed EGFR’s. We assayed the binding of EGF to the ependymal cell surface of the rat lateral ventricle as described in the Materials and Methods section. From the ependymal surface binding assay we found no detectable binding (< 4.5 × 10^3 EGFR’s/cell and 2.2 × 10^3 TR’s/cell) on the surface of a rat ependymal cell monolayer. The estimated number of cells in the rat ependymal cell monolayer was less than the number of cells in the tumor binding assays by at least a factor of 10, limiting the sensitivity of the ependymal surface assay.

### Table 1

<table>
<thead>
<tr>
<th>Tissue</th>
<th>% Specific Binding/100 µg</th>
<th>Estimate of Receptors/Cell†</th>
</tr>
</thead>
<tbody>
<tr>
<td>TE-671</td>
<td>0.95</td>
<td>3.0 × 10^3</td>
</tr>
<tr>
<td>medulloblastoma #1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>medulloblastoma #2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>glioblastoma #1</td>
<td>1.10</td>
<td>3.7 × 10^3</td>
</tr>
<tr>
<td>glioblastoma #2</td>
<td>0.19</td>
<td>1.2 × 10^3</td>
</tr>
<tr>
<td>ependymoma #1</td>
<td>1.76</td>
<td>5.9 × 10^3</td>
</tr>
<tr>
<td>ependymoma #2</td>
<td>0.33</td>
<td>1.1 × 10^3</td>
</tr>
<tr>
<td>ependymoma #3</td>
<td>0.62</td>
<td>4.0 × 10^3</td>
</tr>
<tr>
<td>ependymoma #4</td>
<td>0.77</td>
<td>6.1 × 10^3</td>
</tr>
</tbody>
</table>

* ND = not detected: detectable binding below the sensitivity of the assay.
† Estimate assumes tumors have the same affinity as TE-671 cells.

### Table 2

<table>
<thead>
<tr>
<th>Tissue</th>
<th>% Specific Binding/100 µg</th>
<th>Receptors/Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>TE-671</td>
<td>7.8</td>
<td>2.0 × 10^4</td>
</tr>
<tr>
<td>medulloblastoma #1</td>
<td>0.24</td>
<td>1.4 × 10^4</td>
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<tr>
<td>medulloblastoma #2</td>
<td>0.59</td>
<td>8.2 × 10^3</td>
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<tr>
<td>glioblastoma #1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>glioblastoma #2</td>
<td>0.20</td>
<td>1.9 × 10^3</td>
</tr>
<tr>
<td>ependymoma #1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>ependymoma #2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>ependymoma #3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>ependymoma #4</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* ND = not detected: detectable binding below the sensitivity of the assay.

### Discussion

Growth factors and growth factor receptors have been linked to malignant cell transformation. Epidermal growth factor may be involved in the progression or promotion of tumors. Epidermal growth factor receptors are expressed by a variety of human cancers in cell culture and in surgical samples. Glioblastomas multiforme have been shown by Libermann, et al., 28 to amplify and overexpress the EGFR gene. Whittle, et al., 43 detected EGFR binding in 10 of 14 glioblastomas multiforme, one of nine low-grade gliomas, and five of six meningiomas. Amplification of the EGFR gene was demonstrated by Helseth, et al., 21 in four of 16 glioblastomas, none of six astrocytomas, none of two oligodendrogliomas, one of three mixed gliomas, none of 11 meningiomas, and three of six brain metastases. Gerosa, et al., 15 detected abnormal expression of the EGFR gene in four of five human glioblastoma cell lines. In these same five glioblastoma cell lines, Gerosa, et al., 15 observed overexpression of the N-ras oncogene, and an increased expression of the N-ras proto-oncogene was noted in five of five glioblastoma biopsy samples. Overexpression of Ha-ras has been reported in a human glioblastoma cell line. 23

Ependymoma, primarily a pediatric tumor that often displays malignant characteristics such as their tendency to recur following surgery, radiation therapy, and chemotherapy, expressed significant EGFR levels. The only reported results pertaining to PNET’s by Whittle, et al., 43 and Libermann, et al., 30 demonstrated that EGFR’s were not present on the single tumor that each group studied. Normal brain tissue and tumors often considered benign because of their slow-growing nature (cerebellar astrocytoma and acoustic neurinoma) did not express EGFR’s. These findings are consistent with those reported by Whittle, et al., and Helseth, et al. 21 As in previous reports, we found expression of EGFR on two of four human glioblastoma tissue samples.

A recent report by McKanna and Cohen 32 is interesting in light of the finding of EGFR’s on all four ependymomas. A substrate for the EGFR tyrosine ki-
nase, P35, was localized to a raphe of primitive glial ependymal cells in the central nervous system (CNS) of rat embryos. The P35 calcium- and phospholipid-binding protein appears by embryonic Day 12 before the arrival of ventral commissural axons, and disappears by postnatal Day 5. P35 was not detected in any other areas of the CNS in either rat embryos or adults. 52

The TR mediates iron uptake by cells. 42 Cells undergoing division express more TR's than quiescent cells. The presence of TR's has previously been demonstrated on human breast cancer cells and on a human melanoma cell line. In addition, Zovickian, et al., 44 demonstrated increased expression of TR on glioblastoma and medulloblastoma-derived cell lines and in surgical tissue samples of glioblastoma and medulloblastoma using solid-phase indirect radioimmunoassay techniques. Our data extend the reported results of Zovickian, et al., by quantifying TR on the TE-671 PNET-derived cell line and on medulloblastoma and glioblastoma surgical specimens. Not unexpectedly, "normal" brain tissue that does not divide and slowly growing tumors such as cerebellar astrocytoma and acoustic neuroma did not express detectable levels of TR.

Newer treatment modalities, such as antibody-toxin conjugates, 40 for primary malignant CNS tumors and tumors metastatic to the CNS are being developed. Zovickian, et al., 44 reported a greater than 150- to 1380-fold in vitro selective toxicity of an anti-TR ricin immunotoxin against "target" tumor-derived cells as compared to "non-target" cells. The "target" tissue-culture cells tested were derived from glioblastoma, medulloblastoma, and leukemia. Johnson, et al., 23 reported a 10,000-fold increase in tumor-specific toxicity using an immunotoxin made with CRM 107, a diphtheria toxin mutant with two amino acid changes in the B subunit. 17 CRM 107 linked to an antibody directed against the human TR, 454A12, showed efficient killing of cell lines derived from medulloblastoma, glioblastoma, and breast carcinoma at concentrations between 3.9 × 10−13 and 1.1 × 10−10 M. 23

The complications associated with systemic delivery of immunotoxins can be avoided by direct delivery into a compartmentalized space such as the CSF of the CNS or the abdominal cavity. Intraperitoneal and intrathecal delivery can produce high local concentrations with a greater therapeutic effect. Several investigators have demonstrated in vivo efficacy of immunotoxins in the compartmental treatment of cancer. Watanabe, et al., 42 demonstrated prolonged survival from 30 to 50 days in a nude-mouse model inoculated with 107 human pancreatic adenocarcinoma tissue-culture cells (T3M-4) treated intraperitoneally with 60 μg of an anti-carinoembryonic antigen monoclonal antibody-ricin A-chain immunotoxin at 2, 5, 7, and 10 days after intraperitoneal tumor inoculation. Using Strain 2 guinea pigs, Gregg, et al., 24 extended median survival time by 200% in animals by administering 30 μg/kg intraperitoneal M6-ricin immunotoxin 24 hours after intraperitoneal inoculation of 105 L1210 B-cell leukemia cells. Bjorn and Groetsem, 2 using monoclonal antibodies to the murine TR conjugated to recombinant ricin A-chain, showed prolonged survival in mice inoculated intraperitoneally with P388 D1 lymphoid tumors following intraperitoneal treatment. For leptomeningeal neoplasia, Zovickian and Youle45 demonstrated extended survival in guinea pigs, corresponding to a 2 to 5 log kill of L12 leukemia cells delivered into the cisterna magna, using an anti-idiotype monoclonal antibody (M6)-intact ricin immunotoxin delivered intrathecally 24 hours after tumor inoculation.

The in vitro success of immunotoxins against human brain tumors, glioblastoma multiforme, and medulloblastoma and the in vivo efficacy of immunotoxins against leptomeningeal neoplasia suggest that these agents may be useful against malignant tumors that spread via CSF pathways such as medulloblastoma and ependymoma. The identification of EGFR on glioblastoma and ependymoma and of TR on glioblastoma and medulloblastoma provides a target for antibody-directed immunotoxins and supports the possibility of their use in future clinical trials. Further support to warrant future clinical use of antibody-toxin conjugates is the absence of toxicity after intrathecal administration of high concentrations of CRM 107-based immunotoxins in rhesus monkeys. 23

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Address reprint requests to: Richard J. Youle, Ph.D., Surgical Neurology Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Building 10, Room 5D37, Bethesda, Maryland 20892.

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