Human-specific c-neu proto-oncogene protein overexpression in human malignant astrocytomas before and after xenografting

Jerald J. Bernstein, Ph.D., Anna V. Anagnostopoulos, B.S., Emily A. Hattwick, B.S., and Edward R. Laws, Jr., M.D.

Departments of Neurological Surgery and Microbiology and Immunology, George Washington University School of Medicine, and Laboratory of Central Nervous System Injury and Regeneration, Department of Veterans Affairs Medical Center, Washington, D.C.

Overexpression of human-specific c-neu proto-oncogene transmembrane tyrosine kinase receptor protein (p185) is an index of cell transformation and of poor patient survival in several malignancies. The authors studied this protein in low- and high-grade human malignant astrocytomas before and after xenografting into aspiration pockets in rat cortex. Human-specific p185"*-positive cells were found in tumor specimens from all grades of astrocytoma. Significantly fewer p185"*-positive cells were observed in the low-grade versus the high-grade astrocytomas examined (p < 0.05). Human specific p185"*-positive cells were also positive for the human major histocompatibility complex, human leukocyte antigen (HLA)-DR, as well as glial fibrillary acidic protein and S-100 protein. Fresh suspensions of tumor tissue were prelabeled with the plant lectin Phaseolus vulgaris leukoagglutinin and xenografted into pockets in rat cortex. A class of human p185"*-positive cells found in tissue samples from all grades of astrocytoma migrated in the rat brain along parallel and intersecting nerve fiber bundles and basement membrane-lined surfaces. Migrated p185"*-positive cells were also positive for HLA-DR, Phaseolus vulgaris leukoagglutinin, glial fibrillary acidic protein, and S-100 protein, suggesting that they were in fact human astrocytoma cells. Simultaneous expression of human p185"*, HLA-DR, and glial fibrillary acidic protein was observed in a class of human malignant astrocytoma cells in both tumor tissue and xenografted cells that migrated into rat brain. These molecules are signature proteins for the study of the spread of human malignant astrocytomas in an animal model, and indicate that transformed human malignant astrocytoma cells can migrate within the parenchyma of the central nervous system and could play a role in the development of multifocal tumors.

Key Words • astrocytoma • cell migration • xenograft • oncogene • glioma • rat • human

Although the pattern of spread of human malignant astrocytomas has been extensively studied from pathological material, the method by which the transformed cells ultimately form these patterns is poorly understood. Clinically, malignant astrocytomas are considered to be nonmetastatic, although they tend to extend through the brain along parallel and intersecting white matter tracts and along basement membrane-lined surfaces such as blood vessels. At biopsy, viable tumor cells can be retrieved not only from the central core of the tumor but also from apparently normal human brain tissue as far as 7 cm from the edge of the mass. Not only can malignant astrocytoma cells be obtained at biopsy some distance away from the tumor mass but multiple tumors may be found in different areas of the brain (multifocal tumors). One of the most plausible explanations of multifocal tumor formation is the migration of individual transformed cells which could act as foci for new masses; therefore, the migratory capacity of human malignant astrocytoma cells is of importance.

One of the alternatives to studying autopsy-derived human material in order to gain insight into the migratory pattern of human malignant astrocytomas is to examine an animal model using biopsies from human malignant astrocytomas as xenograft donor tissue. When a biopsy is prelabeled with a plant lectin (Phaseolus vulgaris leukoagglutinin, PHAL) and xenografted into a pocket in the cortex of a rat, the human cells in the aspiration pocket are rapidly killed by a hyperimmune reaction and removed by phagocytic activity of the rat immune system. Anti-PHAL immunohisto-
Oncogene overexpression in astrocytomas

chemistry, however, reveals a zone surrounding the implantation pocket in the rat brain (peritumoral zone) where human malignant astrocytoma cells enter the rat brain and are protected by the rat blood-brain barrier. Cells migrate from this zone through the cortex and along parallel and intersecting nerve fiber bundies and basement membrane-lined structures. When cultured C6 glioma cells (ethylinitrosourea-transformed rat astrocyte cell line) or fetal rat astrocytes are grafted into rat cortex, they show a similar pattern of spread to that observed with xenografted human malignant astrocytoma cells. It appears that there are consistent routes of migration of transformed or fetal cells in the mature central nervous system.

It is advantageous to utilize a human-specific intrinsic cell marker to delineate human malignant astrocytoma cells that have transformed in the cell mass of the tumor and to use this intrinsic cell product to identify human malignant astrocytoma cells that have been neuroxenografted in an animal model. The present study utilized the expression of the human-specific c-neu proto-oncogene transmembrane tyrosine kinase receptor protein (p185) as an index of the transformation of human cells in surgically excised low- and high-grade malignant astrocytomas. This intrinsic human specific cell protein lies in the plasma membrane of the cell and is used as an endogenous marker to identify and study the migration of human malignant astrocytoma cells after xenografting into rat brain. The reliability of p185 as a cell signature protein is also compared with a known successful extrinsic marker, the plant lectin PHAL, to test for the presence of both markers in the same cell. Finally, a series of human and rat antibodies are utilized to determine the tissue and species specificity of the migrated cells and show definitively that they are human malignant astrocytoma cells and not derived from human or rat astrocytes, microglia, or blood-derived cells.

Materials and Methods

Control Groups and Cell Lines

Three rats underwent surgery for aspiration of the cortical pocket only ("operated control group"). The antibodies used in the study were tested in these brains to determine whether the operative manipulation of the brain for the implantation of donor human malignant astrocytoma cells would alter the specificity of cells in the host rat brain. Three other rats were used to show antibody specificity ("normal control group"), and the "lesion-only control group" was used to show the effect of the lesion made for implantation on antibody specificity.

Host Animals

Thirty male Sprague-Dawley rats, each weighing 250 gm, were anesthetized with pentobarbital (Nembutal, 50 mg/kg) and mounted in a head holder. The skin on the head was incised and the periosteum removed over the bregma. The skull was trephined at the bregma just left of the sagittal sinus and the dura was exposed. The dura was incised and a cortical implantation pocket, approximately 5.0 mm in diameter, was aspirated at the border of the frontal and somatomotor cortices, down to approximately layer III.

Donor Tissue

The donor tissue was graded by a pathologist and consisted of fresh excised nodules of human malignant astrocytoma from different patients; the tissue included four astrocytomas grade II, four astrocytomas grade III (anaplastic astrocytomas), and 16 astrocytomas grade IV (glioblastomas multiforme) (Fig. 1). All specimens were coded and the patients were anonymous. The astrocytomas were initially placed in a dextrose-saline solution after excision. Two astrocytomas grade II, three anaplastic astrocytomas, and four glioblastomas were then mechanically disrupted and cell suspensions were incubated in 2.0 mg/ml of PHAL in Eagle's minimum essential medium (MEM) for 1 hour, then rinsed twice in fresh MEM. The astrocytomas were implanted within 4 hours of collection. One tumor from each grade was not incubated in PHAL to act as a control specimen to show the influence of PHAL on p185. Positive p185 cells in these control specimens had the same responses as the cells in the PHAL-incubated donor tissue. Operated control rats were infused with MEM (the vehicle).

Implantation

A heavy cell suspension of astrocytoma cells was aspirated through a Hamilton syringe with a No. 20 unbeveled needle. The astrocytoma cell suspension was dripped into the cortical cavity until the cavity was filled. Five minutes later, the skin was closed with clips and 0.2 ml penicillin was administered intramuscularly.

Tissue Collection

Five host rats each were sacrificed at 1, 2, 3, 7, 14, 21, 30 or 45 days postimplantation. To collect tissue, the host rats were deeply anesthetized with pentobarbital (50 mg/kg); this was followed by an intracardiac perfusion of 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.2). The brain was removed, dehydrated in alcohol, infiltrated and embedded in 54°C paraffin, and cut into 8.0-μm sections.

Immunofluorescence

The sections were deparaffinized, rehydrated, and

*Eagle's minimum essential medium supplied by Meditech, Herndon, Virginia.
rinsed in PBS. After a 30-minute blocking step in PBS containing 5% sheep serum and 1% bovine serum albumin, the sections were incubated overnight at 4°C with human specific anti-p185 neo (mouse monoclonal (Ab3), dilution 1:130; and/or rabbit polyclonal (c-erb B-2), dilution 1:30) and PHAL (rabbit anti-PHAL 1:600) diluted in PBS containing 0.25% Triton X-100, 1% sheep serum, and 0.1% bovine serum albumin. The next day, the sections were rinsed four times in PBS containing 1% sheep serum (PBS-serum), then incubated for 1 hour with combined donkey anti-rabbit immunoglobulin (Ig)G (fluorescein isothiocyanate (FITC)-conjugated) and donkey anti-mouse IgG (rhodamine isothiocyanate (RITC)-conjugated) diluted 1:75 in PBS-serum. After the sections were rinsed four times in PBS-serum, the FITC fluorescence was enhanced by incubation with FITC-conjugated rabbit anti-goat IgG diluted 1:75 in PBS-serum, four rinses in PBS-serum, and incubation with FITC-conjugated goat anti-rabbit IgG§ diluted 1:75 in PBS-serum. The sections were then rinsed four times in PBS and coverslipped with mounting medium. The RITC- and FITC-specific fluorescences were observed with epifluorescence (Tables 1 and 2).

Peroxidase-Antiperoxidase Immunohistochemistry

The procedure for immunofluorescence was as described above except that only one primary antibody (mouse monoclonal anti-p185 neo, rabbit polyclonal anti-p185 neo, or rabbit polyclonal anti-PHAL) and either unconjugated sheep anti-mouse IgG or sheep anti-rabbit IgG was used for each section examined. The

† Mouse monoclonal antibody supplied by Oncogene Science, Manhasset, New York; rabbit polyclonal antibody supplied by Triton Diagnostics, Alameda, California; rabbit anti-PHAL supplied by Vector Laboratories, Burlingame, California.

‡ Donkey anti-rabbit IgG and donkey anti-mouse IgG supplied by Jackson Immunoresearch, Westgrove, Pennsylvania.

§ Rabbit anti-goat IgG and goat anti-rabbit IgG supplied by Jackson Immunoresearch, Westgrove, Pennsylvania.

¶ Sheep anti-mouse IgG and sheep anti-rabbit IgG supplied by Jackson Immunoresearch, Westgrove, Pennsylvania.
Oncogene overexpression in astrocytomas

### TABLE 1

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity</th>
<th>Species, Molecule, or Cell to be Marked</th>
</tr>
</thead>
<tbody>
<tr>
<td>p185&lt;sup&gt;++&lt;/sup&gt;-encl(R)</td>
<td>− +</td>
<td>human-specific c-neu oncoprotein</td>
</tr>
<tr>
<td>p185&lt;sup&gt;+++&lt;/sup&gt;-encl(H)</td>
<td>− +</td>
<td>rat-specific c-neu oncoprotein</td>
</tr>
<tr>
<td>GFAP, GFAP</td>
<td>+ +</td>
<td>GFAP, astrocytes, &amp; derivatives</td>
</tr>
<tr>
<td>Ab1 (H)</td>
<td>+ +</td>
<td>human-specific HLA-DR reactive molecule, T cells</td>
</tr>
<tr>
<td>S-100 protein</td>
<td>+ +</td>
<td>S-100 protein, macroglia (astroglia)</td>
</tr>
<tr>
<td>HLA-DR(H)</td>
<td>− +</td>
<td>human-specific MHC reactive molecule, macrophages, T cells</td>
</tr>
<tr>
<td>LCA or OX-1(R)</td>
<td>− −</td>
<td>membrane glycoprotein on rat-specific leukocytes</td>
</tr>
<tr>
<td>DAKO-LCA(H)</td>
<td>+ +</td>
<td>human-specific macrophages</td>
</tr>
<tr>
<td>MCA-342(R)</td>
<td>+ −</td>
<td>mature rat-specific macrophages</td>
</tr>
<tr>
<td>BMA-2569(H)</td>
<td>− +</td>
<td>human-specific macrophages</td>
</tr>
<tr>
<td>OX-42(R)</td>
<td>+ ‡ ‡</td>
<td>rat-specific MHC macrophages</td>
</tr>
<tr>
<td>OX-18(R)</td>
<td>+ ‡ ‡</td>
<td>rat-specific MHC</td>
</tr>
<tr>
<td>PHAL</td>
<td>− − ‡</td>
<td>non-specific plant lectin premarker, PHAL</td>
</tr>
</tbody>
</table>

*Manufacturer-claimed specificity may not include cross-species testing.*

**Antigens supplied by Oncogene Science, Manhasset, New York; DAKO Corp., Carpinteria, California; Chemicon, El Segundo, California; BioProducts for Science, Inc., Indianapolis, Indiana; Accurate Chemical, Westbury, New York; and Vector Laboratories, Burlingame, California.

### TABLE 2

<table>
<thead>
<tr>
<th>Antibody*</th>
<th>Positive Specificity</th>
<th>Negative Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>p185&lt;sup&gt;+++&lt;/sup&gt;-encl</td>
<td>++ + +</td>
<td>− −</td>
</tr>
<tr>
<td>GFAP</td>
<td>+ + + +</td>
<td>+ + +</td>
</tr>
<tr>
<td>S-100 protein†</td>
<td>++ + +</td>
<td>++ +</td>
</tr>
<tr>
<td>HLA-DR(H)‡</td>
<td>++ + +</td>
<td>++ +</td>
</tr>
<tr>
<td>leukocyte(H)</td>
<td>− − + +</td>
<td>+ +</td>
</tr>
<tr>
<td>macrophage(H)</td>
<td>− + + +</td>
<td>− +</td>
</tr>
<tr>
<td>PHAL§</td>
<td>++ + +</td>
<td>++ +</td>
</tr>
</tbody>
</table>

*Abbreviations: GFAP = glial fibrillary acidic protein; HLA = human leukocyte antigen; MHC = major histocompatibility complex; LCA = leukocyte common antigen; PHAL = Phaseolus vulgaris lectoglaucinulin; + = weak positivity; ++ = moderate positivity; +++ = intense positivity; − = negative for the antibody.

† S-100 protein is found in astrocytes and other macroglia.

‡ Anti-HLA-DR is the antibody to the human major histocompatibility complex.

§ Dispersed fresh astrocyoma cells are incubated with PHAL, a plant lectin (2 μg/ml for 1 hr) prior to implantation. The endocytosed lectin is identified by a PHAL-specific antibody.

### Staining Controls

Negative controls for immunohistochemistry were incubated with pre-immune serum instead of primary antibody or the primary antibody incubation step was eliminated. Controls for cross-fluorescence were also included. Sections were reacted with rabbit anti-GFAP and then incubated with FITC-conjugated sheep anti-rabbit IgG and observed under FITC optics or incubated with RITC-conjugated sheep anti-rabbit IgG and observed under RITC optics. An additional control was used with each antibody to determine if any antibody was human or rat cross-reactive (Tables 1 and 2). Furthermore, human or rat antibody specificity cross-reaction studies were carried out on normal and lesion-only rat brain, normal human brain, and the tumour tissue.

### Cell Measurements

Five slides of each tumor and three slides of each implanted rat brain were immunohistochemically stained with the antibodies (monoclonal and/or polyclonal) for human p185<sup>+++</sup>-encl. The slides were coded and all of the p185<sup>+++</sup>-encl-positive cells in a randomly selected 0.03 sq mm area (10 areas/tumor, 0.15 × 0.22 sq mm/side) were counted. At least one p185<sup>+++</sup>-encl-positive cell was necessary before an area was counted. The decoded data were statistically analyzed using the average number of p185<sup>+++</sup>-encl cells/field/tumor as the independent variable in the Student t-test.

### Results

#### Control Specimens

Negative control specimens stained without the primary antibody had no positive cells, with little to no background staining. Control samples for cross-fluorescence were negative. The FITC filter combinations did

J. Neurosurg. / Volume 78 / February, 1993 243
FIG. 2. Photomicrographs of human-specific p185<sup>imm</sup> immunochemistry showing positive cells in low-grade (astrocytoma grade II) and high-grade (anaplastic astrocytoma and glioblastoma) human malignant astrocytomas and controls. A and B: Few cells were positive in low-grade astrocytomas (arrows). PAP, × 480. C: Normal human cortex control tissue stained for human-specific p185<sup>imm</sup> was negative. PAP, × 400. D and E: Cells in high-grade anaplastic astrocytomas were intensely positive for p185<sup>imm</sup> (arrows). PAP, × 600. F: High-grade human anaplastic astrocytoma control tissue (without primary antibody) was negative for human-specific p185<sup>imm</sup>. PAP, × 600. G and H: Cells in high-grade glioblastoma were intensely positive for p185<sup>imm</sup>. PAP, × 600. I: Lesion-only rat brain tissue does not exhibit human-specific p185<sup>imm</sup>-positive cells. PAP, × 600.

Not fluorescent RITC-conjugated antibodies, and the RITC filter combinations did not fluoresce FITC-conjugated antibodies. Human-specific p185<sup>imm</sup> monoclonal and polyclonal antibodies were indeed human specific since they labeled cells in grade II astrocytomas, anaplastic astrocytomas, and glioblastoma tumors but did not label cells in normal or injured rat brain (Tables 1 and 2). Rat-specific p185<sup>imm</sup> was negative for cells in
Oncogene overexpression in astrocytomas

![Graph showing percentage of cells in human malignant astrocytomas.](image)

**Fig. 3.** Histogram showing the percentage of cells in human malignant astrocytomas that were positive for human-specific p185<sub>neo</sub>. Counts were made from randomly selected sections that had at least one positive cell.

normal and lesion-only rat brain. There were no intrinsic transformed rat cells present or generated by the implantation procedure. Glial fibrillary acidic protein (Ab1) was not human-specific since it cross-reacted with normal rat astrocytes. Two antibodies, OX-18 (rat-specific major histocompatibility complex) and OX-42 (rat-specific macrophages), stained astrocytes in both the human astrocytomas and the rat brain and were not included in the study since they had no cell or species specificity. The human-specific antibodies to leukocyte common antigen and macrophages were only positive for cells in the human tumors (anaplastic astrocytoma and glioblastoma), whereas the rat-specific antibodies for these cells were negative in the human tumors. The human-specific markers for leukocyte common antigen, reactive microglia, and macrophages (HLA-DR) were positive in the human tumors but negative for normal human and normal and lesioned rat brain. Rat-specific antibodies for the same antigens were positive for rare cells in normal rat brain and occasional cells in the lesioned rat brain (Table 2).

When p185<sub>neo</sub> antibodies were used in studies on normal human cortex and edematous human cortex containing reactive astrocytes and normal and lesioned rat cortex, no positively stained cells for p185<sub>neo</sub> (human or rat) were found in the tissue. Although present in normal cells, p185<sub>neo</sub> was only detectable when over-expressed in the astrocytomas (Table 2).

**Astrocytomas**

**High-Grade Glioblastoma.** The pathologist-graded samples of glioblastoma multiforme were confirmed by staining with hematoxylin and eosin (H & E) (Fig. 1). Scattered within these heterogeneous tumors were many islets of astrocytoma cells, some of which were positive for p185<sub>neo</sub> protein (Fig. 2). Although there were many fields that did not contain p185<sub>neo</sub> cells, within the fields that did contain at least one positive cell, 20% to 25% of the cells present expressed p185<sub>neo</sub> (mean 97 total cells/field; range 19.4 to 24.25 p185<sub>neo</sub>-positive cells/field) (Fig. 3). The p185<sub>neo</sub>-positive cells showed intense plasma and cytoplasmic membrane staining as well as cytoplasmic positivity (Fig. 2). The nuclei of positive cells did not stain for p185<sub>neo</sub>. Cells that contained p185<sub>neo</sub> were also positive for GFAP, S-100 protein, HLA-DR, and PHAL (in prelabeled specimens), indicating that p185<sub>neo</sub>-containing cells were human astrocytoma cells (Table 2).

Occasional cells in the tumor were positive for human-specific leukocyte common antigen, macrophages (BMA-25F9), and HLA-DR but were negative for GFAP demonstrating that leukocytes, macrophages, T cells, and microglia were present in the tumor (Table 1). These cells were also negative for human-specific p185<sub>neo</sub>. The p185<sub>neo</sub>-positive cells in glioblastoma did not stain with the battery of tested antibodies, indicating that they were not blood- or immune system-derived cells, normal or reactive astrocytes, or microglia (Tables 1 and 2).

**High-Grade Anaplastic Astrocytoma.** There were no discernible differences in the immunohistochemically positive-staining responses of the individual p185<sub>neo</sub> cells in anaplastic astrocytoma and glioblastoma (Fig. 2). Anaplastic astrocytomas were confirmed with H & E stains (Fig. 1). The p185<sub>neo</sub>-positive cells were scattered throughout the field but were not in islets. Again, although there were many negative fields, approximately 20% to 25% of the cells present expressing p185<sub>neo</sub>-positive cells per field when at least one positive cell was observed (mean 79 total cells/field; range 15.8 to 19.75 p185<sub>neo</sub>-positive cells/field) (Fig. 3). Cells that were positive for p185<sub>neo</sub> in anaplastic astrocytomas were also intensely positive for GFAP, S-100 protein, human specific HLA-DR, and PHAL in (prelabeled specimens). The tumors were negative for all rat-specific antibodies utilized. By the same anatomical criteria used with the glioblastoma cells, the p185<sub>neo</sub>-positive cells in anaplastic astrocytomas were human astrocytoma cells and not blood- or immune system-derived cells, normal or reactive astrocytes, or microglia. Similar to glioblastoma, the anaplastic astrocytomas studied contained cells that marked for human-specific leukocyte common antigen and HLA-DR but not with p185<sub>neo</sub> or GFAP (Table 2). These cells were leukocytes, macrophages, T cells, and reactive microglia. Cells in the tumor were negative for the rat-specific antibodies used in the study (Tables 1 and 2).

**Low-Grade Astrocytoma.** The pathologist-graded samples of malignant astrocytomas grade II (Fig. 1) contained cells that showed weak human-specific p185<sub>neo</sub> positivity (Fig. 2). There were very few positive cells scattered as individual cells throughout the tumor, approximately 8% positive cells/field (mean 15 total cells/field; 1.2 p185<sub>neo</sub>-positive cells/field) (Fig. 3). The positivity was in the form of small membrane spheroids in the cytoplasm (perhaps mitochondria) and of beading in the plasma membrane. Those cells that contained p185<sub>neo</sub> were also positive for GFAP, S-100 protein, human-specific HLA-DR, and PHAL (in prelabeled specimens) (Table 2). A few cells in the tumor were also positive for human-specific c-neu leukocyte common antigen and HLA-DR, identifying them as leukocytes, T cells, and reactive microglia (Table 1). These cells
Fig. 4. Photomicrographs showing positivity and negativity after high-grade anaplastic cells were xenografted into host rat cortex and migrated into the corpus callosum. PAP, × 600. A: Human anaplastic astrocytoma cells were intensely positive for human-specific p185 on Day 2 after xenografting. B: Anaplastic astrocytoma cells were intensely positive for glial fibrillary acidic protein on Day 21 after xenografting. C: Normal rat cortex was negative for human-specific p185. D: Human anaplastic astrocytoma cells were positive for S-100 protein on Day 2 after xenografting. E: Human anaplastic astrocytoma cells were positive for the human major histocompatibility complex human leukocyte antigen (HLA)-DR on Day 2 after xenografting. F: Normal rat cortex was negative for human-specific HLA-DR.

were not positive for human-specific p185 or GFAP, indicating that they were not human astrocytoma cells. The tumors were negative for human-specific macrophages (BMA-25F9) and for all rat-specific antibodies used.

Positivity Measurements. The p185\textsuperscript{c-neu} cells were counted in randomly selected fields in the pathological samples of the tumors (0.03 sq mm/field, Fig. 3). To be counted, a field had to contain at least one p185\textsuperscript{c-neu}-positive cell. There were many fields that did not contain a p185\textsuperscript{c-neu}-positive cell so they were not counted or used in the database. In the specimens examined, there were significantly more p185\textsuperscript{c-neu}-positive cells in high-grade tumors than in low-grade tumors (p < 0.05, Fig. 3). The difference in numbers of p185\textsuperscript{c-neu}-positive cells in the two types of high-grade tumors was statistically indistinguishable (p > 0.05, Fig. 3).

Xenografting

High-Grade Astrocytoma Xenograft to Rat Brain. At all time points examined (up to 30 days), the xenografted, migrated, high-grade, human malignant astrocytoma cells were intensely positive for p185\textsuperscript{c-neu} and were also positive for GFAP, S-100 protein, human-specific HLA-DR, and PHAL (Figs. 4 and 5). Immunohistochemistry revealed that the migrated cells were not human or rat blood- or immune system-derived elements, human or rat normal or reactive astrocytes, or human or rat microglia. Human-specific HLA-DR, the human histocompatibility cell-surface antigen was also expressed by the human cells in the rat brain and colabeled in these cells with GFAP (Fig. 6). Human-specific HLA-DR was conserved and was a reliable marker for xenografted human cells. This confirmed that p185\textsuperscript{c-neu}-positive cells in the rat brain were xeno-
Oncogene overexpression in astrocytomas

**FIG. 5.** Photomicrographs showing positivity and negativity after high-grade glioblastoma cells were xenografted into host rat cortex and migrated into the corpus callosum. PAP, × 600. A: Human glioblastoma cells were intensely positive for human-specific p185<sup>calo</sup> on Day 7 after xenografting. B: Human glioblastoma cells were intensely positive for glial fibrillary acidic protein on Day 21 after xenografting. C: Normal rat cortex was negative for human-specific p185<sup>calo</sup>. D: Human glioblastoma cells were positive for S-100 protein on Day 2 after xenografting. E: Human glioblastoma cells were positive for the human major histocompatibility complex human leukocyte antigen (HLA)-DR on Day 21 after xenografting. F: Normal rat cortex was negative for human-specific HLA-DR.

grafted human tumor cells (Figs. 4 and 5). At the same time, PHAL, an extrinsic cell label applied before grafting, stained positively and appeared in HLA-DR-positive xenografted cells (Fig. 6). Thus, human-specific p185<sup>calo</sup> and HLA-DR were reliable intrinsic markers and PHAL was a reliable extrinsic marker since they were retained or expressed after grafting. The preferred migration route was ventral from the implantation pocket through cortical gray matter and into the corpus callosum, then along parallel and intersecting nerve fiber bundles (Figs. 4 and 5). A favored migration path was at the dorsal corpus callosum cortical interface (Figs. 4 and 5). The grafted human cells also migrated along basement membrane-lined surfaces (blood vessels, glia limitans, and subependymal space). In gray matter, they were found in the habenula, hippocampus, and thalamus.

*Low-Grade Astrocytoma Xenograft to Rat Brain.* For the first 7 days, the grafted, migrated human cells were weakly positive for p185<sup>calo</sup> (Fig. 7). The cells were as described for the tumor sample alone (Fig. 1). These cells were also positive for GFAP, S-100 protein, HLA-DR, and PHAL (if incubated), indicating that they were human astrocytoma cells. By Day 14, the xenografted human cells were intensely positive for p185<sup>calo</sup> with highly positive cytoplasm and plasma membrane (Fig. 7). Immunohistochemistry revealed that the migrated cells were not human or rat blood- or immune system-derived elements, normal or reactive astrocytes, or microglia. The intensely stained p185<sup>calo</sup> human cells were also positive for GFAP, S-100 protein, HLA-DR, and PHAL. Although the migration path was as described above, there were considerably fewer cells; this was most probably because of the low number of transformed cells in the original tumor donor tissue (Fig. 2).

**Discussion**

The data presented here show that a class of cells in high- and low-grade human malignant astrocytomas
expresses the human-specific c-neu oncoprotein. This oncoprotein is not expressed in sufficient quantities to be detected by immunohistochemistry in normal or edematous human brain. However, a class of low- and high-grade astrocytoma cells showed positive staining for this protein, suggesting that it is overexpressed in these cells. The positive astrocytoma cells also retained the overexpression after xenografting in rat brain.

After cell suspensions of fresh human malignant astrocytoma tissue are xenografted into rat cortex, the human-specific p185<sup>neo</sup>-expressing human astrocytoma cells migrate from the cell suspensions of the tumor into the rat brain<sup>5,6</sup> and continue to express the human-specific p185<sup>neo</sup>. As far as could be determined from immunohistochemistry, the human-specific p185<sup>neo</sup> cells were also positive for human-specific HLA-DR, as well as GFAP, S-100 protein, and PHAL (if preincubated). The p185<sup>neo</sup>-positive cells appear to be the only migrating human cells in the rat brain. The presence of the human-specific c-neu proto-oncogene protein, the presence of GFAP, human-specific HLA-DR, and PHAL in the migrated cells, and the negative results from the other antibodies used demonstrated that the migrated cells in the rat brain were human graft-derived astrocytoma cells and not human or rat normal or reactive astrocytes, microglia, macrophages, reticular cells, or leukocytes. The presence of human-specific p185<sup>neo</sup> and HLA-DR in donor human malignant astrocytoma cells can be used as a specific marker for the location of these transformed cells in pathological studies and after xenografting. The specificity of these intrinsic human proteins makes the animal xenograft model a powerful experimental tool in the study of human malignant astrocytomas.

The p185<sup>neo</sup> cells that migrate from the human malignant astrocytomas into rat brain joined the reactive astrocytes at the border of the lesion pocket to create what might be equivalent to the "peritumoral zone"<sup>5,6</sup> observed on diagnostic imaging studies (magnetic resonance imaging and computerized tomogra-
Oncogene overexpression in astrocytomas

Fig. 7. Photomicrographs showing positivity and negativity after low-grade astrocytoma (grade II) cells were xenografted into host rat cortex and migrated into the corpus callosum. A: Astrocytoma grade II cells were weakly positive for human specific p185<sup>c-neu</sup> 3 days after implantation. PAP, x 400. B: Astrocytoma grade II cells were intensely positive for glial fibrillary acidic protein 14 days after implantation. PAP, x 600. C: Normal rat cortex was negative for human specific p185<sup>c-neu</sup>, PAP, x 600. D: Human astrocytoma grade II cells were positive for S-100 protein on Day 14 after xenografting. PAP, x 600. E: Astrocytoma grade II cells were also positive for the human major histocompatibility complex human leukocyte antigen (HLA)-DR (arrow) on Day 2 after implantation. PAP, x 400. F: Normal rat cortex was negative for human specific HLA-DR. PAP, x 600.

These data indicate that the astrocytoma cells that are human-specific p185<sup>c-neu</sup>-positive are the malignant and migratory cells in the tumor. The same cells were also positive for the human major histocompatibility complex HLA-DR and for GFAP, S-100 protein, and exogenously applied PHAL (if incubated). The p185<sup>c-neu</sup>-positive cells in high-grade astrocytomas showed both cytoplasmic and plasma membrane staining. Other studies performed in breast adenocarcinomas have reported cytoplasmic protein staining in association with a 155-kD protein and have hypothesized that this might be a neu-like cross-reacting protein, another c-neu product derived from alternate splicing, or a mitochondrial membrane protein. However, in the breast cancer studies, cytoplasmic positivity did not indicate malignancy due to cytoplasmic expression in negative carcinomas. The brain biopsies studied here showed cytoplasmic staining in high-grade astrocyto-
mas and positive cytoplasmic spheroids in low-grade astrocytomas but no cytoplasmic positivity in normal or edematous human brain. Further studies including Western blots need to be performed to characterize both the plasma membrane and cytoplasmic proteins.

There is a known correlation between malignancy, prognosis, and a poor patient survival rate and the overexpression of p185
\(^{\text{onc}}\) in malignant breast, endometrial, ovarian, pancreatic, gastric, and lung tumors. Our studies indicated a distinct difference in the numbers of p185
\(^{\text{onc}}\)-positive cells in high-grade versus low-grade astrocytomas (20% to 25% vs. 8%). The survival time of patients with high-grade astrocytomas is approximately 1 year and with low-grade astrocytomas approximately 5 years; therefore our findings might suggest a correlation between the overexpression of p185
\(^{\text{onc}}\) and patient prognosis. However, a significantly larger number of cases and an examination of long-term survival would further substantiate the prognostic significance of c-neu receptor overexpression.

Since oncogenes and proto-oncogenes act in concert to transform cells definitively, it must be stressed that the presence of p185
\(^{\text{onc}}\) alone is only one indication that the astrocytoma cell is malignant. We should expect that other proto-oncogenes are amplified in human malignant astrocytomas if the cell is transformed. Other studies have shown that n-myc and v-sis are amplified in human astrocytomas. In a previous report on a single recurrent high-grade astrocytoma, there were excessive amounts of messenger ribonucleic acid (mRNA) that hybridized to their homologous proto-oncogenes n-myc and v-sis, which was probably due to gene amplification (n-myc 80 copies, v-sis 3 to 4 copies). Gene amplification and mRNA accumulation may be indicators of tumorigenicity and pathogenesis and may also be accompanied by increased levels of oncoprotein and RNA. It follows that gene amplification studies need to be performed to further evaluate the role of c-neu in tumorigenicity and survival rate in patients with astrocytomas.

Acknowledgments

The authors thank Dr. B. Ammerman, Dr. J. D. Cooney, Dr. B. Guthrie, Dr. T. Chappell, and the residents of the Department of Neurological Surgery, George Washington University School of Medicine for the astrocytomas used in this study.

References

Oncogene overexpression in astrocytomas


Manuscript received February 19, 1992.
Accepted in final form June 19, 1992.
This research was supported by National Institutes of Health National Cancer Institute Grant CA48956 and the Department of Veterans Affairs to Dr. Bernstein.
Part of this research was submitted by Anna V. Anagnostopoulou as a dissertation in partial fulfillment of the Ph.D. requirements at George Washington University.
Address reprint requests to: Jerald J. Bernstein, Ph.D., Laboratory of Neuro-Oncology, 705 Ross Hall, George Washington University School of Medicine, Washington, D.C. 20037.