Fibronectin and Glial Fibrillary Acidic Protein Expression in Normal Human Brain and Anaplastic Human Gliomas

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ABSTRACT

The expression of fibronectin (FN), an extracellular matrix glycoprotein with adhesive and oposcinic properties, was examined in normal and neoplastic human central nervous system tissues. In both normal adult and fetal brain, FN expression, as detected by immunohistochemistry, was restricted to the vasculature. Neurons and glia did not stain. FN expression in nine of 10 gliomas examined was qualitatively similar to that in normal fetal and adult brain in that it was confined to the blood vessels. Nevertheless, in one case, there was interstitial expression of FN. Four tumors exhibited varying degrees of fibroblastic overgrowth in which cells containing FN were detected. Frozen sections of eight athymic mouse-borne anaplastic human gliomas were stained simultaneously by a double-label fluorescein-rhodamine fluorescence technique for both FN and glial fibrillary acidic protein (GFAP), a glial and glioma marker. Seven of eight tumors expressed FN, seven of eight expressed GFAP, and six of eight expressed both. FN was detected as fine fibrils and specks between cells while GFAP presented as a homogeneous intracellular fluorescence. In many areas of the tumors, groups of cells expressed both FN and GFAP. Eleven human glioma-derived cell lines were examined for both FN and GFAP expression. In two cell lines, there were cells which contained both GFAP and FN simultaneously. Athymic mouse-borne tumors were developed by the injection of an FN-producing glioma-derived cell line into athymic mice. Human FN plasma levels were monitored, and some tumors larger than 1.0 g produced FN which was detectable in the plasma, thereby demonstrating that tumor-produced FN could be used to monitor tumor growth in vivo. FN also may be important in the clearance of gram-positive bacteria (2, 35). Transformation often reduces or eliminates FN production and expression in cultured cells (11, 19, 40, 41, 45, 47), and a loose correlation has been reported between tumorigenicity, anchorage independence, and a lack of FN expression (5, 14).

In this study, we have examined the expression of FN in normal and neoplastic CNS tissues. Because the expression of FN by a given cell type can change with environment, i.e., solid tumor versus cell culture (5), we have examined surgical biopsies, primary and established glioma-derived cell lines, and athymic mouse-borne tumors to determine if FN expression is in any sense phase specific in glioma-derived systems.

The expression of a universally accepted biochemical differentiation marker of astrocytes, GFAP, was examined with FN expression. GFAP is considered astrocyte specific in normal and reactive tissues and generally glioma restricted in neoplastic tissues (6, 8). It is expressed in some glioma-derived cell lines (1) and in athymic mouse-borne tumors derived from anaplastic human gliomas (13).

We found that FN expression in normal adult and fetal human brain and in glioblastomas is restricted to blood vessels. One glioblastoma contained FN not only in the blood vessels but also in interstitial areas containing tumor cells. Six of 8 athymic mouse-grown anaplastic human gliomas expressed both FN and GFAP and 2 anaplastic human glioma-derived cell lines expressed both simultaneously. Studies to determine if human tumor-produced FN could be used to monitor tumor growth in athymic mice carrying human gliomas demonstrated that mice carrying tumors weighing greater than 0.1 g often have human FN in their plasma.

MATERIALS AND METHODS

Maintenance and Growth of Cell Culture Lines. Human fetal lung fibroblasts (WI-38) and human cell lines derived from anaplastic gliomas (D-54 MG, D-171b MG, D-171d MG, U-251 MG, U-251 MG sp, and U-251 MG cl1, cl2, and cl3), a meningioma (D-187), an osteogenic sarcoma (2T), and a gliosarcoma (D-173 MG) were maintained in culture in 100-mm Petri dishes (Falcon Plastics) containing ZO (Grand Island Biological Co.) supplemented with 20% fetal calf serum, 584 mg glutamine per liter, and 50 μg gentamicin sulfate per ml. For testing, 3- x 3-mm coverslips were placed in the Petri dishes prior to cell plating; once cells reached confluence, the coverslips were removed from the dishes.

Establishment of Cell Culture Lines from Human Anaplastic Gliomas Grown in Athymic Mice. Human anaplastic gliomas growing...
in athymic BALB/c mice were excised under sterile conditions and minced in serum-free medium (ZQ) to yield 1.0-cm pieces which were placed in a trypsinization flask containing a 0.05% collagenase solution (Worthington Biochemical Corp.) at 37°. The flask contents were stirred gently for 5 min; large pieces of the tumor were allowed to settle out, the supernatant was removed and centrifuged at 250 × g, and the cells were stored on ice. This procedure was repeated twice, and all 3 supernatant lots were combined and placed in a 100-mm Petri dish containing ZQ supplemented with 20% fetal calf serum.

Absorption and Testing of Goat Anti-Human FN Serum. Aliquots of goat anti-human FN serum prepared as described by Ruoslahti et al. (33) were rendered nonreactive with mouse and bovine FN by absorption with mouse and bovine serum proteins. Lyophilized goat anti-FN serum was rehydrated to a concentration of 80 mg/ml, and dilutions of the antisera were tested in an immunofluorescence assay against normal BALB/c mouse brain and spleen and a human glial tumor grown in athymic mice. Mouse serum proteins, also from BALB/c mice, were obtained by retroorbital bleedings; the blood was maintained at 37° during clotting to reduce FN binding to fibrinogen (31). Mouse serum, 1:1, also absorbed with normal bovine serum served as a negative serum control. Frozen sections of bovine liver were stained with the unabsorbed and absorbed sera to test the effectiveness of the absorption.

This technique for antibody absorption was repeated with another 1.5-ml aliquot of 80 mg goat anti-human FN serum per ml using bovine serum proteins (160 mg/ml) for the absorption. Before absorption, very little cross-reactive anti-bovine activity was detected in immunofluorescence assays with sections of bovine liver. After 3 absorptions with 12 mg of bovine serum protein per absorption, no anti-bovine activity was detectable, and the goat anti-human FN serum was absorbed with bovine serum proteins a final time.

Absorption of Rabbit Anti-human FN Serum to Remove Cross-reactivity with Bovine Serum. Affinity-purified rabbit anti-human FN antibody (33) was absorbed exhaustively to remove any cross-reactivity with bovine serum proteins. Lyophilized antibody was diluted to a concentration of 1 µg/ml in normal bovine serum, incubated for 1 hr at 25°, and centrifuged at 100,000 × g for 60 min. A saturated (27%) ammonium sulfate fraction of normal rabbit serum (ammonium sulfate: water, 1:1) also absorbed with normal bovine serum served as a negative serum control. Frozen sections of bovine liver were stained with the unabsorbed and absorbed sera to test the effectiveness of the absorption.

Generation and FITC Labeling of Swine Anti-Goat IgG Antibody. Thirty ml of saturated (27%) ammonium sulfate were added drop by drop to 30 ml of normal goat serum, and the resulting immunglobulin-enriched fraction was further enriched by DEAE A-50 Sephadex column chromatography (7, 23). The resulting goat globulin was used to hyperimmunize a 30-kg swine. After immunization, a saturated (27%) ammonium sulfate fraction of the hyperimmune serum (ammonium sulfate: serum, 1:1) was made, and the globulin solution was further enriched by DEAE A-50 Sephadex column chromatography (7). FITC was then conjugated to this globulin protein (10); unbound FITC was removed by Sephadex G-25 column chromatography (12). The labeled protein was placed on a DEAE A-50 Sephadex chromatography column (7) and eluted with a discontinuous phosphate- NaCl buffer (12). The FITC:protein ratio of the conjugate was determined spectrophotometrically (10), and those fractions containing protein with a FITC:protein ratio of 2.0 were pooled and used at a concentration of 2.5 mg/ml.

Fluorescent Staining of Frozen Sections and Tissue Culture Cells. Both the 8-µm frozen sections and the 3- x 3-mm coverslips bearing cell monolayers were fixed with -70° acetone for 30 sec and then washed 2 times for 5 min each time with DPBS. The sections or cells were then incubated at 25° for 30 min with primary antibody (rabbit anti-human GFAP serum, 1:10; or goat anti-human FN serum, 1:100) or normal serum and DPBS controls. After three 5-min washes, FITC-conjugated secondary antibody (Cappel Laboratories; 1:16) (goat anti-rabbit IgG serum or rabbit anti-goat IgG serum depending on the species of primary antibody) was added, and the sections or cells were again incubated for 30 min at 25°. The specimens were mounted in 50% DPBS:50% glycerol and viewed under a Zeiss universal microscope with FITC interference (500 nm) and No. 50 barrier filters. Frozen sections were used because attempts to stain conventionally prepared formalin-fixed paraffin-embedded tissues were unsuccessful even when sections were subjected to trypsin or pepsin digestions (4) prior to antibody application.

The double-staining technique which allowed visualization of both GFAP and FN in the same specimen was accomplished by the use of secondary antibody generated in a third species. The secondary antibody for the primary rabbit anti-human GFAP serum was a rhodamine-conjugated swine anti-rabbit IgG (Dako Corp.; 1:8) while the secondary antibody for the goat anti-human FN serum was an FITC-conjugated swine anti-goat IgG (2.5 mg/ml). In 2 control preparations, the anti-GFAP serum was replaced with DPBS and with normal rabbit serum. No rhodamine-conjugated anti-rabbit IgG bound and only FITC fluorescence was observed. In 2 other control preparations, the anti-FN serum was replaced with DPBS and normal goat serum. In this case, only rhodamine fluorescence was observed. These controls established that the 2 secondary antisera were not binding to one another and each was binding only to the species of IgG against which it was directed. These double-stained preparations were viewed with a Zeiss universal microscope first with FITC interference (500 nm) and No. 50 barrier filters and then with rhodamine interference (546 nm) and No. 58 barrier filters.

Peroxidase-Antiperoxidase Staining for FN. The peroxidase-antiperoxidase technique used to stain FN (38) was modified from an already-described protocol (13). Briefly, 8-µm frozen sections were rehydrated with 10% normal goat serum to reduce nonspecific binding of the secondary antibody, washed 3 times with DPBS, and incubated with the primary antibody (affinity-purified rabbit anti-human FN, 1 mg/ml; or a saturated ammonium sulfate fraction of normal rabbit serum, 1 mg/ml) for 2 hr at 37°. The slides were again washed in DPBS and immersed in anhydrous methanol with 0.3% H2O2 to block any endogenous peroxidase activity. The blocking step was delayed until after primary antibody exposure to avoid FN denaturation prior to antibody binding. After a washing, the slides were incubated with secondary goat anti-rabbit IgG serum (1:40) for 30 min at 25°, washed, and incubated with rabbit peroxidase-antiperoxidase (Starnberger and Meyer, Inc.) for 30 min at 25°. After washing, the slides were subjected to a 7-min incubation with diaminobenzidine solution (Sigma Chemical Co.) (5 mg diaminobenzidine in 10 ml 0.05 M Tris-HCl, pH 7.6, and 5 µl 30% H2O2). The reaction was stopped by immersion in delonized H2O, and the slides were counterstained with an aqueous solution of 1% methyl green, dehydrated through a graded alcohol series to xylene, and mounted.

Detection of Human FN in the Plasma of Anaplastic Human Glioma-bearing Athymic Mice. Tumors induced by the injection of U-118 MG into athymic mice were used in the fifth serial mouse passage (7). Thirty µl of mechanically dispersed tumor were injected into the flank s.c. space of 35 athymic mice. After 10 days, 13, 16, and 19, 5 mice were killed on Day 22, and 4 were killed on Days 25 and 28. Four animals were lost to disease. In all cases, citrated heart blood samples were taken and plasma samples were frozen at -70° until the end of the study when a previously described double-antibody competition radioimmunoassay (33) was used to determine human FN levels in the plasma samples.
RESULTS

Immunoperoxidase Staining of FN in Normal Adult Human Brain, Fetal Human Brain, and Anaplastic Human Gliomas. Frozen sections of 2 fetal (16 and 22 weeks) and 2 adult human brains were stained immunohistochemically for the presence of FN. In all cases of both adult and fetal tissues, the capillary walls contained substantial amounts of reaction product. Positive staining ended abruptly at the edge of the capillaries and all neurons and glia were negative. The walls of arteries found in the adult brain tissue were stained markedly. FN was observed throughout these walls and was not restricted to the luminal surfaces (Fig. 1; Table 1).

Ten human glioblastomas were stained similarly, and FN was present in all types of blood vessels found in the tumors (Fig. 2) while areas that were clearly tumor as judged by comparison with adjacent hematoxylin and eosin-stained sections were negative except in one case (120) where a mesh-like network of fibrillar reaction product coursed between and around tumor cells. Fine punctate deposits of FN were also occasionally associated with the fibrillar FN (Fig. 3). Four tumors showed areas of intense perivascular fibroblastic proliferation. In 3 tumors, this fibrous tissue was FN positive while the bordering tumor was negative (Fig. 4), and in one tumor (120), the tumor tissue was also positive (Table 2).

Immunofluorescent Staining of FN and GFAP in Primary and Established Cell Lines. Five primary and 5 established human cell lines were stained for FN expression by indirect immunofluorescence using an anti-FN serum that is not reactive with bovine FN. All 5 primary cell lines, WI 38 (fetal lung fibroblasts), D-187 (meningioma derived), D-173 MG (gliosarcoma derived), D-171b MG, and D171d MG (both glioma derived), and one established cell line, U-118 MG (glioma derived), showed significant amounts of FN staining. The pattern of FN expression was fibrillar with the fibrils forming an extracellular mesh-like network between and over the cells.

Table 1
In situ expression of FN in normal adult and fetal human brain: an immunoperoxidase study

<table>
<thead>
<tr>
<th>Blood vessels</th>
<th>Neurons and glia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal adult human brain</td>
<td></td>
</tr>
<tr>
<td>A77137</td>
<td>++++*</td>
</tr>
<tr>
<td>A80281</td>
<td>++</td>
</tr>
<tr>
<td>Normal fetal human brain</td>
<td></td>
</tr>
<tr>
<td>F40359</td>
<td>++</td>
</tr>
<tr>
<td>N61866</td>
<td>+++</td>
</tr>
</tbody>
</table>

* ++ -- +++, intensity of staining. --, failure to stain.

Table 2
In situ expression of FN in anaplastic human gliomas: an immunoperoxidase study

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Blood vessels</th>
<th>Tumor tissue</th>
<th>Perivascular proliferation</th>
</tr>
</thead>
<tbody>
<tr>
<td>91</td>
<td>++ + + + +</td>
<td>--</td>
<td>NA*</td>
</tr>
<tr>
<td>120</td>
<td>++ + + + +</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>165</td>
<td>++ + + + +</td>
<td>--</td>
<td>NA</td>
</tr>
<tr>
<td>583</td>
<td>++ + + + +</td>
<td>--</td>
<td>NA</td>
</tr>
<tr>
<td>667</td>
<td>++ + + + +</td>
<td>--</td>
<td>NA</td>
</tr>
<tr>
<td>692</td>
<td>++ + + + +</td>
<td>--</td>
<td>NA</td>
</tr>
<tr>
<td>702</td>
<td>++ + + + +</td>
<td>--</td>
<td>++</td>
</tr>
<tr>
<td>703</td>
<td>++ + + + +</td>
<td>--</td>
<td>NA</td>
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<tr>
<td>735</td>
<td>++ + + + +</td>
<td>--</td>
<td>NA</td>
</tr>
<tr>
<td>788</td>
<td>++ + + + +</td>
<td>--</td>
<td>NA</td>
</tr>
</tbody>
</table>

* ++ -- +++, intensity of staining. --, failure to stain. NA, not applicable; no fibroblastic perivascular proliferation was found in sections studied.

Punctate areas of positive staining were noted around many of the nuclei and represented either intracytoplasmic or substratum deposition of FN (Fig. 5). The remaining cell lines, 2T (osteogenic sarcoma derived), D-54 MG, U-251 MG, and U-251 MG sp (glioma derived), were negative for FN. The CNS tissue-derived cell lines were also stained for GFAP expression; D-54 MG, D-171b MG, D-171d MG, D-173, D-187, and U-118 MG were negative for GFAP while in U-251 MG and U-251 MG sp, GFAP was detected. FN and GFAP expression were not seen together in the same cell line (Table 3).

Simultaneous Immunofluorescent Staining of FN and GFAP in Athymic Mouse-borne Anaplastic Human Gliomas. Frozen sections of 8 anaplastic gliomas serially transplanted in athymic mice were subjected to a double-label rhodamine-fluorescein fluorescence technique so FN and GFAP could be visualized in the same tissue section. The anti-FN serum used was absorbed with mouse serum proteins and did not react with mouse FN. Seven of 8 tumors contained FN, 7 of 8 contained GFAP, and 6 of 8 contained both. FN expression was present as very fine fibrils and specks between cells while GFAP appeared to be within cells usually producing a homogeneous fluorescence in these sections rather than the characteristic fibrillary pattern seen commonly in attached cultured cells. Any given area of a tumor could be negative for both, positive for either, or positive for both (Table 4). Two tumors (Tumors 120 and 391) showed areas that clearly demonstrated expression of both FN and GFAP (Fig. 6).

Simultaneous Immunofluorescent Staining of FN and GFAP in a Cell Line Derived from an Athymic Mouse-borne Human Anaplastic Glioma. Athymic mouse-borne tumor 120 was placed in cell culture because both FN and GFAP were detected in frozen sections. The cultured cells were stained for both FN and GFAP; approximately 75% of the cells were positive for GFAP while 20% expressed FN. Although they constituted only about 10% of the total cells, cells positive for both FN and GFAP were present (Fig. 7). The fibrillar pattern of GFAP expression common to other GFAP-positive cell lines was present in this cell line while the FN pattern of expression was mostly of the punctate variety consistent with intracytoplasmic or substratum expression. The extracellular strial pattern was, however, not uncommonly seen with the stria attached to GFAP-positive cells.

Immunoperoxidase Staining of an Established Anaplastic Human Glioma-derived Cell Line and Its Clones. Coverslips carrying cell monolayers of U-251 MG and 3 single-cell clones, U-251 MG cl1, cl2, and cl3, were stained for both FN and GFAP. All 4 cell lines expressed marked amounts of GFAP in >95% of their cells. The parent line (U-251 MG) and 2 of the clones (cl1 and cl3) expressed no detectable amounts of FN. U-251 MG cl2, however, expressed moderate amounts of FN in the typical strial pattern (Fig. 8).

Detection of Human FN in the Serum of Anaplastic Human Glioma-bearing Athymic Mice. Levels of human FN in the plasma of athymic mice which were carrying actively growing human glioma-derived (U-118 MG) tumors were measured. By staining with immunofluorescence, sections of these tumors were demonstrated to contain FN. Four of 31 mice tested had detectable levels of human FN in their plasma. Two of 4 of the mice had tumors weighing more than 2.0 g and had human FN plasma levels of 2.1 and 5.8 μg/ml. The third and fourth mice carried tumors weighing 0.19 and 1.25 g and had human FN...
FN and GFAP Expression

Table 3
Immunofluorescent staining of FN and GFAP in primary and established human cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>Passage level</th>
<th>FN</th>
<th>GFAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>WI 38</td>
<td>Fetal lung</td>
<td>20</td>
<td>++</td>
<td>ND*</td>
</tr>
<tr>
<td>D-17 1b MG</td>
<td>Glioma</td>
<td>4</td>
<td>++++</td>
<td>-</td>
</tr>
<tr>
<td>D-1714 MG</td>
<td>Glioma</td>
<td>3</td>
<td>++++</td>
<td>-</td>
</tr>
<tr>
<td>D-173 MG</td>
<td>Gliosarcoma</td>
<td>9</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>D-187</td>
<td>Meningioma</td>
<td>2</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>2T</td>
<td>Osteosarcoma</td>
<td>462</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>D-54 MG</td>
<td>Glioma</td>
<td>74</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>U-116 MG</td>
<td>Glioma</td>
<td>561</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>U-251 MG</td>
<td>Glioma</td>
<td>538</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>U-251 MG sp</td>
<td>Glioma</td>
<td>46</td>
<td>-</td>
<td>++++</td>
</tr>
</tbody>
</table>

* + — ++, intensity of staining. —, failure to stain. ND, not done.

Table 4
FN and GFAP expression as detected by immunofluorescence in human gliomas grown in athymic mice

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Passage level</th>
<th>FN</th>
<th>GFAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>91</td>
<td>5</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>112</td>
<td>5</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>114</td>
<td>5</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>115</td>
<td>5</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>120</td>
<td>3</td>
<td>++</td>
<td>++</td>
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<tr>
<td>123</td>
<td>3</td>
<td>++</td>
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<tr>
<td>241</td>
<td>4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>391</td>
<td>3</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* + — ++, intensity of staining. —, failure to stain.

Studies of FN distribution within the CNS can be affected by several factors. FN expression in other cell types has been shown to be transformation sensitive (11, 41, 45), cell cycle sensitive (25), and dependent on cell environment (i.e., in vivo or in vitro) (5). Changes in assay sensitivity can also cause an apparent change in FN expression. These sources of variation are important factors in evaluating FN expression in the CNS.

Immunoperoxidase staining of frozen sections of normal adult and fetal brain in this study revealed FN only in the walls of blood vessels. Schachner et al. (36) studied paraformaldehyde-perfused nonprimate vertebrate CNS tissue with immunocytochemical electron microscopy and found staining only of the luminal surface of endothelial cells. The authors suggest that this FN may be adsorbed from the plasma. In an immunofluorescent study at the light microscopic level, Paetau et al. (26) found FN patterns consistent with both vessel lumen and basement membrane distribution. Our results also demonstrate the presence of FN in the vessel wall and do not support the claim of a luminal distribution only as made by Schachner et al. (36).

Paetau et al. (26) also studied FN in human malignant gliomas and found marked staining of the vasculature including the glomeruloid vascular cell proliferations characteristic of glioblastoma. They also found some faint fluorescence in areas of tumor. Our study also demonstrated FN in the walls of all types of both normal and reactive blood vessels, and in one case (120), positive staining was detected in areas containing tumor cells. Paetau et al. (26) suggest this staining results from leakage of plasma FN into the tumor through breaches in the blood-brain barrier. Because such breaches have been documented (43) and because plasma-borne FN (cold-insoluble globulin) and cell surface FN have been shown repeatedly to be indistinguishable in heterologous antiserum-based assays (17, 18, 44), any FN stained in the tumors could be either hematogenous or tumor in origin. The transplantation of the tumors into athymic mice and the use of a human FN specific antiserum allowed us to study only tumor FN and not plasma FN. The detection of FN in 7 of 8 tumors and the close association of FN and GFAP expression in 6 of 8 demonstrated that glial tumor cells are capable of FN expression. This question of mutual expression of FN and GFAP also has been pursued in cell culture systems.

Four studies with cultured neonatal rat CNS cells (20, 27, 30, 39) concluded that GFAP and expression are not seen together, and Raff et al. (30) stated that FN was expressed in the CNS only by fibroblasts and leptomeningeal cells. Kennedy et al. (16) performed a similar study with fetal human brain and received similar results. Kavinsky and Garber (15), however, studied embryonal mouse brain cultures and found extensive FN expression by GFAP-positive cells. Our studies of cultured cells include primary and established cell lines derived from human gliomas. Six contained FN, 6 contained GFAP, and one contained neither as determined by immunohistochemistry. Two cell lines were stained positively for both. The data demonstrate the heterogeneous nature of FN expression among glioma-derived cell lines. The fact that other authors (20, 27, 30, 39) have not found simultaneous expression of FN and GFAP in CNS tissue-derived cell cultures is not contradictory to our data. The cell lines we have studied were derived from neoplastic tissues and have been serially passed in culture while the cells in other studies were primary explant cultures derived from normal tissues. The limited number of established GFAP-positive cell cultures (1) has made studies of GFAP and FN expression in transformed cells difficult, but this situation may improve with the recent generation of a larger number of

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**Discussion**

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Four studies with cultured neonatal rat CNS cells (20, 27, 30, 39) concluded that GFAP and expression are not seen together, and Raff et al. (30) stated that FN was expressed in the CNS only by fibroblasts and leptomeningeal cells. Kennedy et al. (16) performed a similar study with fetal human brain and received similar results. Kavinsky and Garber (15), however, studied embryonal mouse brain cultures and found extensive FN expression by GFAP-positive cells. Our studies of cultured cells include primary and established cell lines derived from human gliomas. Six contained FN, 6 contained GFAP, and one contained neither as determined by immunohistochemistry. Two cell lines were stained positively for both. The data demonstrate the heterogeneous nature of FN expression among glioma-derived cell lines. The fact that other authors (20, 23, 27, 30, 39) have not found simultaneous expression of FN and GFAP in CNS tissue-derived cell cultures is not contradictory to our data. The cell lines we have studied were derived from neoplastic tissues and have been serially passed in culture while the cells in other studies were primary explant cultures derived from normal tissues. The limited number of established GFAP-positive cell cultures (1) has made studies of GFAP and FN expression in transformed cells difficult, but this situation may improve with the recent generation of a larger number of
GFAP-positive glioma-derived cell lines (46).

Vaheri et al. (42) and Bigner et al. (1) analyzed a number of well-characterized glioma-derived cell lines. Vaheri et al. (42) found that 2 of 5 cell lines contained FN when measured by immunofluorescence, but all 5 were positive when cell extracts were analyzed by the more sensitive radioluminoassay. They, however, did not concurrently study GFAP expression in these cell lines. Bigner et al. (1) found 14 of 15 permanent human anaplastic glioma-derived cell lines contained detectable levels of FN as measured by radioluminoassay. Two cell lines (U-251 MG and U-251 MG sp) contained low but measurable amounts of FN, and immunofluorescent analysis revealed both cell lines also contained marked amounts of GFAP. It was in a clone of U-251 MG that we demonstrated by peroxidase immunohistochemistry both FN and GFAP. The cell culture data in our study demonstrate that human cells of astroglial origin as defined by the presence of GFAP are capable of FN expression. While the radioluminoassay techniques of Vaheri et al. (42) and Bigner et al. (1) are capable of quantifying soluble protein, the immunohistochemical methods used in this study revealed both the perinuclear pattern of fluorescence consistent with intracytoplasmic FN expression and the strial fibrillar pattern so characteristic of the FN extracellular matrix and also allowed the localization of FN to GFAP-positive cells.

Several studies have followed FN secretion in vivo. Saba et al. (34) measured both bioassayable (opsonic) and immunoreactive plasma FN during growth of Sarcoma 180 in mice. Immunoreactive FN paralleled tumor growth while bioassayable FN levels were too low to be useful as a tumor burden monitor with intraperitoneal and pleural fluids of cancer patients. MAD-2 appears to be a fragment of FN, and levels were usually increased above those of nonneoplastic controls, but whether the tumor was the source of the protein was unclear. We chose U-118 MG as the cell line for our study of the feasibility of using FN to monitor athymic mouse-borne tumor burden because it has the highest level of FN synthesis of the well-characterized glioma-derived cell lines proven to be tumorigenic in athymic mice (1, 3). Four of 31 mice had detectable levels of human FN in their plasma; 3 of 4 had tumors which weighed 1.0 g or more. These results show that some tumor FN can gain access to the blood, but levels were too low to be useful as a tumor burden monitor with this particular tumor. Possible explanations include a limited ability of FN to cross into the blood stream, a change in FN synthesis and/or shedding after heterotransplantation to and serial passage in athymic mice and the possibility that the mouse may be acting as a human FN "sink." Oh et al. (24) have demonstrated that human FN injected into the blood stream of mice enters the mouse tissues and assumes a distribution identical to endogenous mouse FN.

Having demonstrated that GFAP-positive cells derived from anaplastic human gliomas could express FN, we then analyzed FN expression as one element in the heterogeneous phenotypes seen with gliomas as a group. Only one in 10 surgical biopsies expressed FN, but after serial passage in athymic mice, 7 of 8 tumors were FN positive. This reversal may be the result of 2 phenomena. In theory, the phenotype of athymic mouse-borne tumors may evolve with passage; selective forces may positively or negatively influence the survival and growth of cellular subpopulations. On the other hand, the environment in which a tumor exists rather than tumor progression may be sufficient to alter phenotype (37). In this study, the change in environment from human brain to mouse s.c. space may induce the tumor to initiate or cease FN synthesis and expression. For example, Chen et al. (5) studied FN expression in tumorogenic virally transformed rodent cells and found that 6 of 19 cell lines were FN positive by immunofluorescence but 18 of 19 of the cell lines were FN negative when grown as solid tumors. When the tumors were returned to culture, FN expression resumed. This demonstrated that the change was not due to an overgrowth of FN-negative cells but rather a cessation of FN expression and deposition by previously FN-positive cells and represents a change in tumor behavior not induced by selection. Although some authors (16, 27, 30) have claimed that GFAP and FN expression are mutually exclusive, this study demonstrates that they are not. They can be seen together in the same cell. This simultaneous expression in both athymic mouse-borne anaplastic human gliomas and in anaplastic human glioma-derived cell cultures supports the thesis that FN expression is seen in transformed glia. Expression of these 2 molecules in anaplastic human gliomas should be considered random with regard to one another until and unless a correlating factor connecting them is demonstrated.

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REFERENCES


Fig. 1. Frozen sections of human brain stained by the peroxidase-antiperoxidase technique for FN. In A, the walls of these capillaries in normal fetal brain are positive (arrow) while neurons and glia do not stain. × 425. In B, FN distribution in normal adult brain is also restricted to the blood vessels (arrows). × 425. In C, this artery in normal adult brain has FN throughout its wall. × 425.

Fig. 2. Peroxidase-antiperoxidase stain of FN in a frozen section of glioblastoma 702. The walls of all types of blood vessels (arrows) in this tumor contain FN. × 100.

Fig. 3. Serial frozen sections of glioblastoma 120 stained by H & E and by peroxidase-antiperoxidase for FN. In A, an area containing tumor cells is shown. H & E, × 425. In B, a serial section stained for FN shows a mesh-like network of fibrillar reaction product (large arrow) coursing around cells. Punctate deposits of (small arrow) are also present. × 425.
Fig. 4. Serial frozen sections demonstrating perivascular fibroblastic proliferation and FN expression in Tumor 692. In A, perivascular fibroblastic proliferation coursing around nests of tumor cells (arrows) are shown. H & E, × 100. In B, these fibroblastic proliferations are stained markedly for FN (arrows) while surrounding tumor tissue is negative. Light methyl green counterstain, × 100. In C, an area of marked fibroblastic proliferation (arrows) rests between nests of tumor cells. H & E, × 100. In D, fibroblastic tissue is positive for FN in this peroxidase-antiperoxidase stain (arrows); tumor cells are negative. Light methyl green counterstain, × 100.

Fig. 5. In this anaplastic human glioma-derived cell line (171d MG) stained by indirect immunofluorescence for FN, both the fibrillar extracellular network (double arrows) and the punctate perinuclear (single arrow) patterns of expression are demonstrated. × 425.
Fig. 6. Frozen sections of athymic mouse-borne anaplastic human gliomas stained simultaneously in the same section by rhodamine-fluorescein double indirect immunofluorescence for FN and GFAP. In A, most cells in tumor 120 are positive for GFAP as demonstrated by intense rhodamine fluorescence. × 425. In B, the same section is also rich in FN as revealed by the presence of fluorescein fluorescence. × 425. In C, tumor 391 has one area rich in GFAP while other cells are negative. × 425. In D, FN is seen in the same area as the GFAP. × 425.

Fig. 7. Rhodamine-fluorescein double indirect immunofluorescent staining of the same cells of tumor 120 in culture. In A, this cell (arrow) is rich in GFAP. × 425. In B, the same cell is also expressing FN (arrow). × 425. In C, 2 cells contain GFAP (arrows). × 425. In D, the 2 cells in C, among others, also stain for FN (arrows). × 425.
Fig. 8. Anaplastic human glioma-derived cell line (U-251 MG cl.) stained by the peroxidase-antiperoxidase technique. In A, greater than 95% of the cells contain GFAP. Methyl green counterstain, × 425. In B, the fibrillar FN forms a network which runs between and over the cells (arrows). Methyl green counterstain, × 425.
Fibronectin and Glial Fibrillary Acidic Protein Expression in Normal Human Brain and Anaplastic Human Gliomas

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