Journal of Neuro-Oncology **24**: 267–280, 1995.

Laboratory Investigation

Products of cells from gliomas: IX. Evidence that two fundamentally different mechanisms change extracellular matrix expression by gliomas

Paul E. McKeever,[1] James Varani,[1] Stephen M. Papadopoulos,[2] M. Wang[1] and J. Philip McCoy[3]
The Department of [1] Pathology and [2] Surgery, The University of Michigan Medical School, Ann Arbor, MI 48109, USA; and the Department of [3] Pediatrics, The University of Medicine and Dentistry of New Jersey, Camden, NJ 08103, USA

Key words: cell lineage, collagen, extracellular matrix, gene expression, glioblastoma, glioma

Summary

Four human astrocytic gliomas of high grade of malignancy were each evaluated in tissue and *in vitro* for percentages of cells expressing glial fibrillary acidic protein (GFAP), collagen type IV, laminin and fibronectin assessed by immunofluorescence with counterstaining of nuclear DNA. Percentages of cells with reticulin and cells binding fluorescein-labeled *Ulex europaeus* agglutinin were also assessed. In tissue, each extracellular matrix (ECM) component was associated with cells in the walls of abnormal proliferations of glioma vessels, and all four tumors had the same staining pattern. Two strikingly different patterns of conversion of gene product expression emerged during *in vitro* cultivation. (1). In the most common pattern, percentages of all six markers consistently shifted toward the exact phenotype of mesenchymal cells in abnormal vascular proliferations: increased reticulin, collagen type IV, laminin and fibronectin; markedly decreased glial marker GFAP and absent endothelial marker *Ulex europaeus* agglutinin. The simplest explanation of this constellation of changes coordinated toward expression of vascular ECM markers is that primary glioma cell cultures are overgrown by mesenchymal cells from the abnormal vascular proliferations of the original glioma. These cell cultures were tested for *in situ* hybridization (ISH) signals of chromosomes 7 and 10. Cells from one glioma had diploid signals. Cells from the other glioma had aneuploid signals indicating they were neoplastic; however, their signals reflected different numerical chromosomal aberrations than those common to neoplastic glia. (2). The second pattern was different. Cells with ISH chromosomal signals of neoplastic glia retained GFAP, and gained collagen type IV. Their laminin and fibronectin diminished, but persisted among a lower percentage of cells. Cloning and double immunofluorescence confirmed the presence of individual cells with glial and mesenchymal markers. A cell expressing GFAP in addition to either fibronectin, reticulin or collagen type IV is not a known constituent of glioblastoma tissue. This provides evidence of a second mechanism of conversion of gene expression in gliomas.

Introduction

The capacity of malignant astrocytic gliomas to elaborate and modify their extracellular matrix (ECM) is a fundamental property which may be directly relevant to glial–stromal interactions related to tumor cell infiltration and tumor angiogenesis. A distinct aspect of glioma cells is their ability to change their expression of their ECM and glial gene products [1–3]. As gliomas progress from low to high grade of malignancy *in situ*, ECM associated with vascular and mesenchymal components increases in structures commonly called endothelial proliferations [3–9]. The glial product GFAP often

is reduced with increased grade [10]. One of the most malignant gliomas, the gliosarcoma contains an increased mesenchymal component which is clearly malignant in certain patients [11–13]. These progressions have been called 'mesenchymal drift'. Cultivation of gliomas *in vitro* accelerates similar changes in the mesenchymal ECM constituent, fibronectin, and in GFAP [14–18]. A better understanding of the mechanisms that cause mesenchymal drift could provide insight into these fundamental properties of malignant astrocytic gliomas.

Two different mechanisms that would explain mesenchymal drift are: 1) a single cell type with highly variable expression of these gene products [19–21] and 2) two different types of cells, one of which outgrows the other [17, 18, 21–23]. A difference between these two possibilities is a constraint imposed by the second mechanism upon the direction of changes in antigenic phenotype. If a cell type overgrows another cell type, the resulting phenotypic changes should maintain a *lineage-consistent direction*. That is, if the overgrowing cell type is a cell of specific lineage within the original glioma tissue, the changes should reflect an alteration toward the phenotype of this lineage.

In contrast, the first mechanism of variable gene product expression would not necessarily produce such a consistent change in the pattern of the phenotype. Bi-lineal, rather than mono-lineal, alterations might be possible. There is little quantitative information about alterations in expression of a group of gene products that cross major cell lineage boundaries. However, cloning of a single human glioma cell line has shown bi-directional alterations in expression of a group of neuroectodermal markers [20].

The present study was designed to test the question whether changes occur in a group of ECM components and cell lineage markers between tissue and early primary culture *in vitro* of human gliomas. If so, would the pattern of changes provide clues regarding the mechanism underlying these changes?

Materials and methods

Glioma tissues

The gliomas used in this study were graded by the neuropathologist (PEM) using criteria of the World Health Organization [5, 24]. Tissue samples for cell culture and cryostat sectioning were taken from the same fresh 0.05–0.2 cm^3 fragment of glioma tissue bisected under sterile conditions. One half was minced for culture and the other half was mounted on a cryostat chuck, sectioned at 7 microns and fixed for 10 min in 100% methanol [4]. Starting material was limited by the need for adequate diagnostic material on these primary biopsies. Autopsy samples of human skin and brain tissue were controls.

Cell cultures

Standard techniques were used for glioma cell culture as described previously [16, 25]. Primary glioma cell cultures were used as early as possible within experimental requirements for duplicate samples grown in special media for six different marker studies. The average passage available was passage four.

Three established human glioma cell lines and cultured fibroblasts were controls. One line (LM) was an established human glioma line from frozen stock in passage 165. The LM line has been characterized previously [26, 27]. The extensively characterized U138 MG human glioma [16, 28] was obtained from the American Type Culture Collection, Rockville, MD. The GFAP+ U251MG human glioma line was obtained as frozen stock in passage 305 [29]. Human fibroblasts were cultured from skin taken at circumcision or at skin biopsy.

Cells were fed and grown on sterile microscopic slides in 100 mm diameter culture dishes in medium supplemented with twice daily additions of 0.25 mM sodium ascorbate (Sigma, St. Louis, MO) as described [29, 30]. After 9 days, the cells were rinsed and fixed for 10 min in 100% methanol. Cells to be stained for fibronectin were grown in serum from which fibronectin was removed by passing it over an affinity column of immobilized gelatin [31]

and were then stained according to the described procedures.

Silver stain for collagen

The Tibor pap silver stain for reticulin was performed as previously described [16, 32]. It is one of two fundamentally different histopathologic approaches to assess numbers and location of cells expressing extracellular matrix (ECM) in gliomas. This approach detects the histochemical capacity of glycosylated ECM fibrils to reduce and deposit silver. Other assays described below depend upon specific binding of fluorescent markers, providing opportunity for corroboration by different ECM assays. Classical 'reticulin' is often attributed to type III collagen, although other types of collagen or proteoglycans might contribute to material identified as collagen by silver staining [33, 34].

Primary antisera

Rabbit anti-laminin and rabbit anti-type-IV collagen sera were donated by Drs. H. Kleinman and G. Martin [35, 36]. Affinity-purified rabbit and anti-laminin antibodies were prepared from whole antiserum by column chromatography as described previously [16]. The purified antibody was aliquoted and stored as stock solution at approximately 1 mg/ml. The purity of the antibody was assessed by SDS-gel electrophoresis and immunoelectrophoresis, and the specificity of the antibody was determined by immunodiffusion, ELISA, and immunoperoxidase staining reactions. Rabbit anti-GFAP was obtained from Dako.

The antibody and antiserum activities were tested in localizing components of human skin and brain tissue and cell line controls. Optimal dilutions of each primary antiserum were also determined at this time. Anti-laminin (1:20), anti-fibronectin (1:50) and anti-type IV collagen (1:20) stained vessels, basement membranes and meninges. These antisera also stained LM, U138MG, and fibroblast cell lines. Anti-GFAP (1:50) stained brain parenchyma (most intensely in subpial astrocytes) and the U251MG glioma cell line.

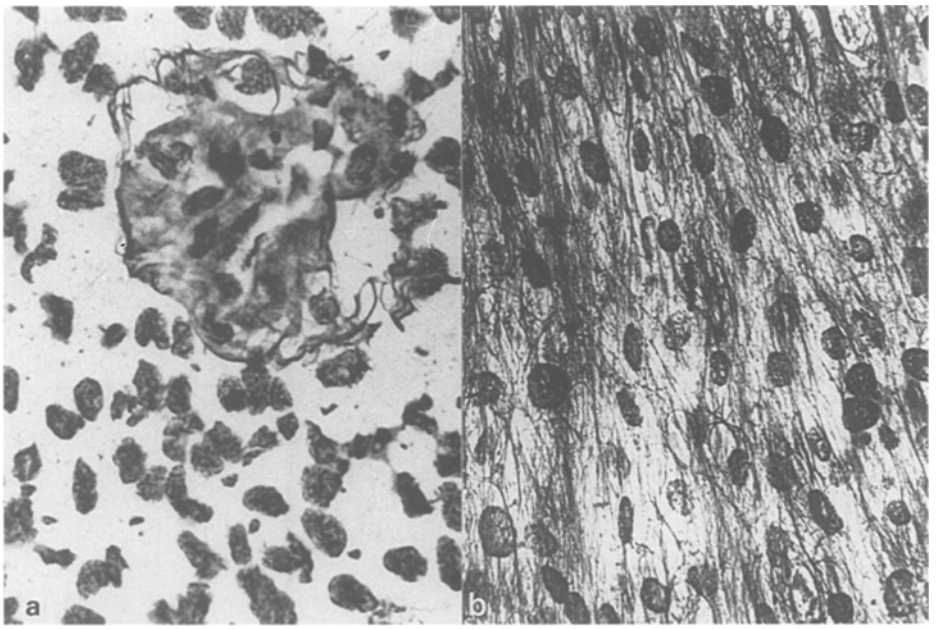
Detection of extracellular matrix protein and GFAP with immunofluorescence

Methanol-fixed specimens were permeabilized with 0.3% Saponin in Dulbecco's PBS with 1 mM EGTA for 15 min to maximize staining of cytoplasmic epitopes. After 3 rinses in 0.01 M phosphate-buffered 0.85% saline (PBS), pH 7.2 (Difco, Detroit, MI), specimens were incubated with primary antiserum for 30 min at room temperature. After 3 rinses in PBS, the specimens were stained with fluorescein-conjugated goat anti-IgG matched to the species of the primary antiserum (Cappel, Malvern, PA) diluted 1:100 in PBS for 30 min. Nuclei were counterstained with the DNA fluorochrome 4′,6-diamidino-2-phenylindole as described below.

Control incubations included substitution of irrelevant antibody of the same species for the primary antibody [4, 16]. Fibronectin staining specificity was tested by substitution of directly labeled irrelevant primary antibody for the fluoresceinated anti-fibronectin and by absorption of anti-fibronectin with pure fibronectin (Collaborative Research, Lexington, MA) [31]. Collagenase digestion provided a further control on specificity of staining for reticulin and collagen type IV. Slides of cellular monolayers were divided with grease into two regions, one of which was incubated 1 day at 37° C with purified collagenase (Form III, Advance Biofactures, Lynbrook, NY) in 0.025 M Tris buffer with 0.01 M calcium chloride. The other region on each slide was incubated in buffer alone. Tissue controls were done on additional sections from the same block.

Lectin-binding ligand fluorescence

Specimens were triple-rinsed with 0.005 M phosphate-buffered 0.15 M saline at a pH 7.2 (PBS) (Biofluids, Rockville, MD). Then 50 μg per ml PBS of fluorescein-conjugated *Ulex europaeus* agglutinin (Sigma, St. Louis, MO) was applied to these



Figs 1–3. Features of tissue and primary *in vitro* cultures of glioma numbers 1–3. These represent the common pattern of gene product expression evident in this study.
Fig. 1. Reticulin and nuclei were stained by silver reduction in tissue (A) and primary culture (B) (original magnification: A, × 665; B, × 410).

cells, which were not allowed to dry during staining. Specimens were incubated for 30 min.

Lectin activity was tested in localizing components of brain tissue and cultured cells. *Ulex europaeus* agglutinin stained capillaries and the endothelial portions of larger vessels. LM, U138MG and fibroblast cells were negative.

Simultaneous detection of antigen with immunofluorescence and nuclear DNA with DAPI fluorochrome

To avoid any possibility of interference from RNA fluorescence and avoid cross-excitation with the primary ECM fluorochrome marker, the DNA fluorochrome 4′,6-diamidino-2-phenylindole (DAPI, Monsanto, Indian Orchard, MA) was used to counterstain nuclei simultaneously stained for ECM. This facilitated quantitation of positive and negative cells. DAPI is a nuclear fluorochrome which stains DNA but not RNA [17]. It does not fluoresce when illuminated with 440–490 nm blue light used to excite fluorescein-labeled markers, and therefore did not interfere with evaluation of ECM components.

After immunofluorescent or lectin staining, the specimens were rinsed 3 times with PBS. They were counterstained with 20 μg/ml DAPI, rinsed in distilled water, and mounted under coverslips in Gelvatol.

Table 1. Extracellular matrix, glycoprotein and GFAP in cells located *in situ* in tissues of human gliomas

Substance	Localization *in situ*	
	Vessel walls	Parenchyma
Reticulin	+	–
Collagen type IV	+	–
Laminin	+	–
Fibronectin	+	–
Ulex europaeus ligand	+ (endothelium)	–
GFAP	–	+

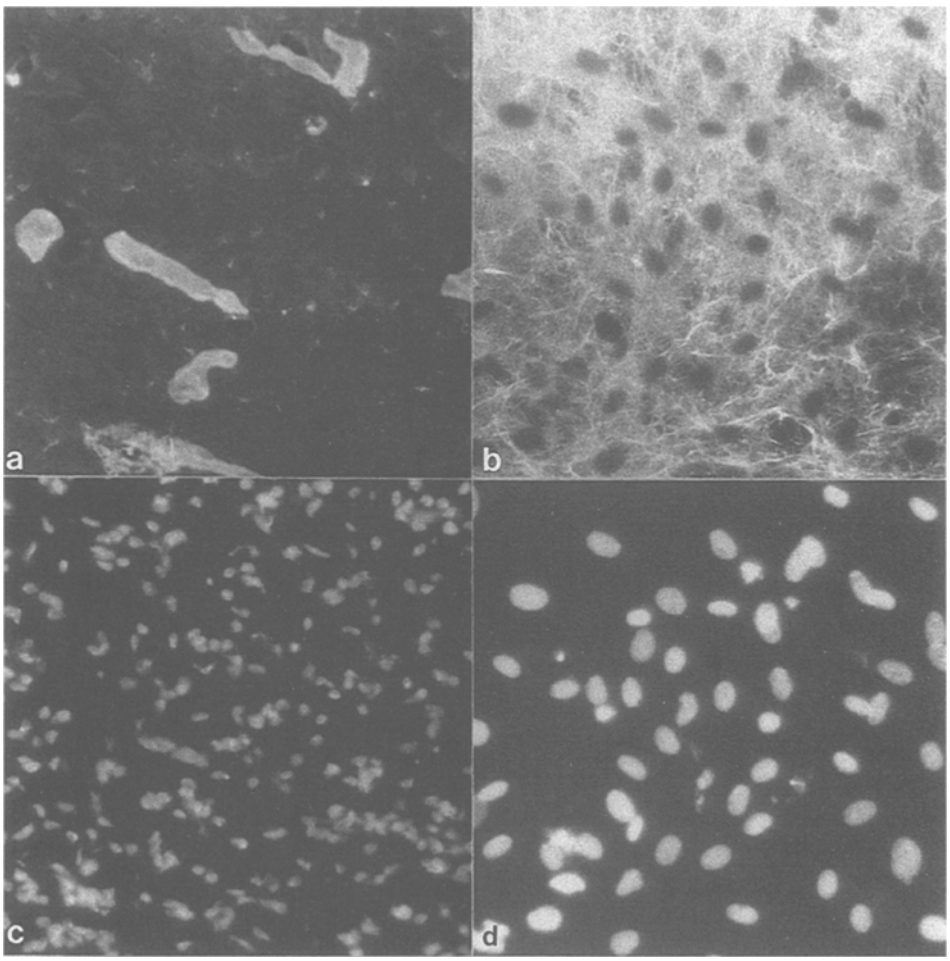


Fig. 2. Collagen type IV stained by indirect immunofluorescence was evident in tissue vasculature (A) and primary culture (B). Nuclei in same field of tissue (C) and culture (D) are counterstained with DAPI (original magnification × 275).

Quantitation of positive and negative cells

The experiments focused upon decisive markers of ECM components with unequivocally positive and negative regions in glioma tissue to facilitate standardization and comparison with these components in cells cultured from the same gliomas. Microscopic fields were selected by random movement of the specimen on the stage [37]. Positive and negative regions of tissue provided internal standardization of levels of fluorescence and silver staining. The lectin stain and antisera to ECM were chosen for this study on the basis of their ability to stain these positive regions brightly with low background fluorescence of negative regions. This produced preparations in which positive and negative cells would be unequivocally interpreted and counted. Cellular fluorescence was considered positive when it was distinctly brighter than background fluorescence of negative controls (brain parenchyma and U251 for fibronectin, brain parenchyma for laminin and type IV collagen, and brain vasculature and fibroblasts for GFAP).

At least 10 microscopic fields per specimen were photographed with Kodak Tri-X film in a Zeiss fluorescence microscope with epi-illumination and transmitted incandescent light optics. For immunofluorescence, images of the same microscopic field were photographed first under 440–490 nm excitation of fluorescein-labeled anti-ECM antibody or lectin (510 nm reflector and LP520 plus KP560 barrier filters), and second under 365 nm excitation of

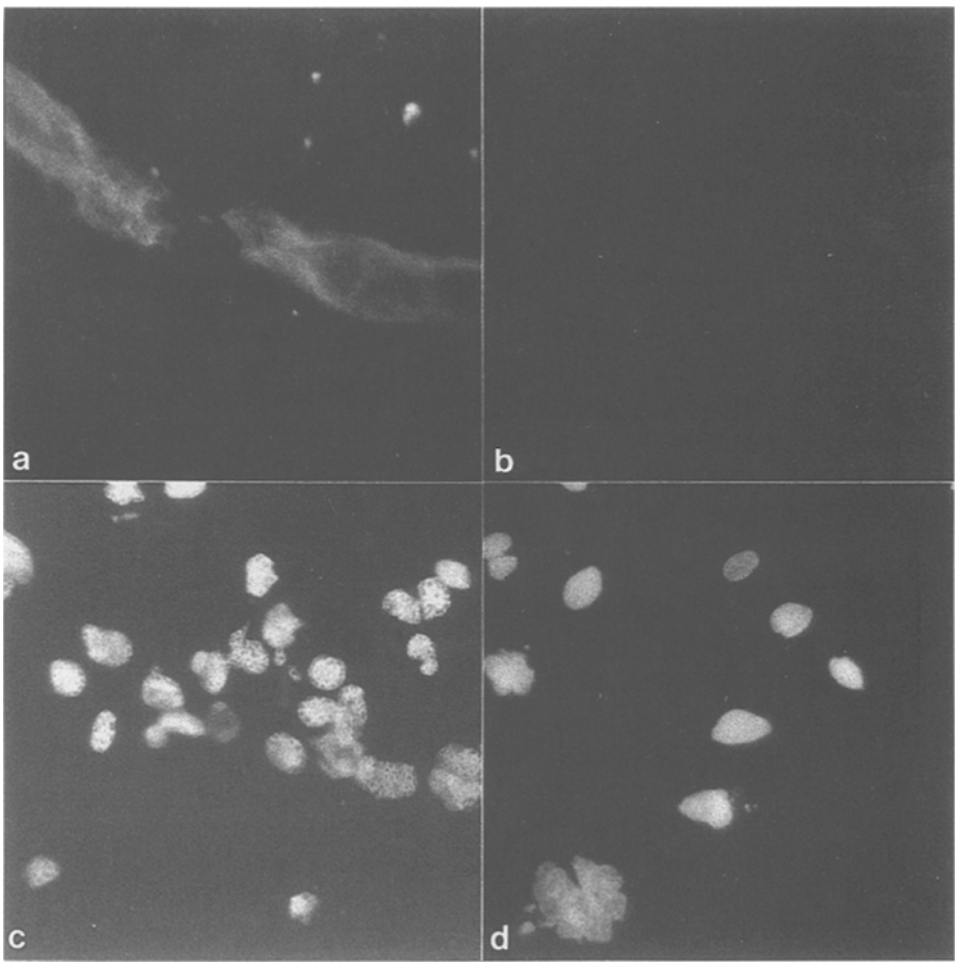


Fig. 3. Binding of fluorescein conjugated *Ulex europaeus* by vascular endothelium in tissue (A) and lack of binding by cells in primary culture (B). Nuclei in same field of tissue (C) and culture (D) were counterstained with DAPI. Micronuclei are evident (original magnification: A and C, × 665; B and D, × 275).

DAPI-stained nuclei (395 nm reflector). For subsequent enumeration, these images were superimposed to determine which nuclei were from positive cells and which were from negative cells.

Since cellular margins were impossible to distinguish with certainty in glioma tissue, cellular nuclei were counted in place of cells. For brevity these determinations are hereafter termed positive and negative 'cells'. On silver-stained preparations the nuclei of positive and negative cells were counted directly with transmitted light optics. The silver stain lacked sufficient cytoplasmic signal to count positive cells. Therefore, cells surrounded or covered by silver fibrils were considered positive. The exact criteria for counting cells positive with fluorescent markers were immunoreactive cytoplasm surrounding the shadow of a negative nucleus confirmed by superimposition of DAPI-stained images of the same field (for example, Figs 2B and 2D). Fluorescent markers that stained cytoplasm sufficiently to reveal this nuclear shadow of absent protein precursor were selected to enable counting of positive cells. This excluded certain markers that failed to meet these criteria from the final data analysis (i.e., other collagen types and *Ricinus communis* agglutinin) that did not produce sufficient cytoplasmic signal for counting.

More than 11,000 cells were counted for this study. A 2 × 2 contingency table was constructed with two rows for the total number of positive and

Table 2. Average percentages of cells that express extracellular matrix, endothelial and glial gene products

Substance	Average percentage of positive cells[a] ± SD		
	In situ	*In vitro*[b]	
Reticulin	32 ± 4	93[c] ± 6	[38]
Collagen type IV	29 ± 8	77[c] ± 25	[64[c]]
Laminin	33 ± 4	69[c] ± 20	[10]
Fibronectin	26 ± 5	98[c] ± 3	[5]
Ulex europaeus ligand	19 ± 6	0[d]	[0 [d]]
GFAP	72 ± 7	6[c] ± 5	[86]

[a] At least 10 microscopic fields from each of three gliomas. [b] The average *in vitro* percentages reflect gliomas 1–3 only, since glioma 4 differed from these. Glioma 4 *in vitro* percentages are in brackets to the right. [c] Significant difference from *in situ* value ($p < 0.05$) by chi-squared analysis. [d] Indistinguishable from background fluorescence of negative controls (brain parenchyma & fibroblasts for *Ulex europaeus*).

negative cells and two columns for the *in situ* and *in vitro* cells. The tabulated data on each ECM component of each glioma were evaluated by Chi-squared tests [38]. These data were expressed as percentages of positive cells (positive cells/total cells multiplied by 100) in Table 2.

Interphase cytogenetics by in situ *hybridization*

Cells grown on glass microscopic slides were fixed in 100% ethanol and air-dried. Specimens were incubated for 30 minutes in 2 × SSC pH 7.0 prewarmed to 37° C. After dehydration and drying from ethanol, specimens were denatured in 70% Formamide/2 × SSC, at 70° C for 2 min. After dehydration in graded ethanols at 4° C, specimens were incubated with 15 ng of heat denatured digoxigenin labeled alpha satellite probes (Oncor) for the chromosomes of interest in 30 µl of hybridization mixture for 0.5–16 h. The hybridization mixture contained 65% formamide, 2 × SSC, 5% dextran sulfate, and 0.1 µg/µl salmon sperm DNA [39–41].

Specimens were washed with 40 ml of prewarmed 0.25X SSC pH 7.0 at 72° C for 5 min, and in 1 × PBD for 2 min. They were then incubated with either fluorescein- or peroxidase-labeled anti-digoxigenin antibody at 37° C for 5 min or 30 min, respectively, and washed 3 times in 1 × PBD at room temperature. Peroxidase-labeled specimens were developed with diaminobenzidine and counterstained with methyl green [41, 42], dehydrated and mounted in Permount. Fluorescein-labeled specimens were counterstained with 0.3 µg/ml propidium iodide and mounted with Gelvatol or glycerol plus 1.4% di-azo-bicyclo-(2,2,2)-octane [43, 44].

Results

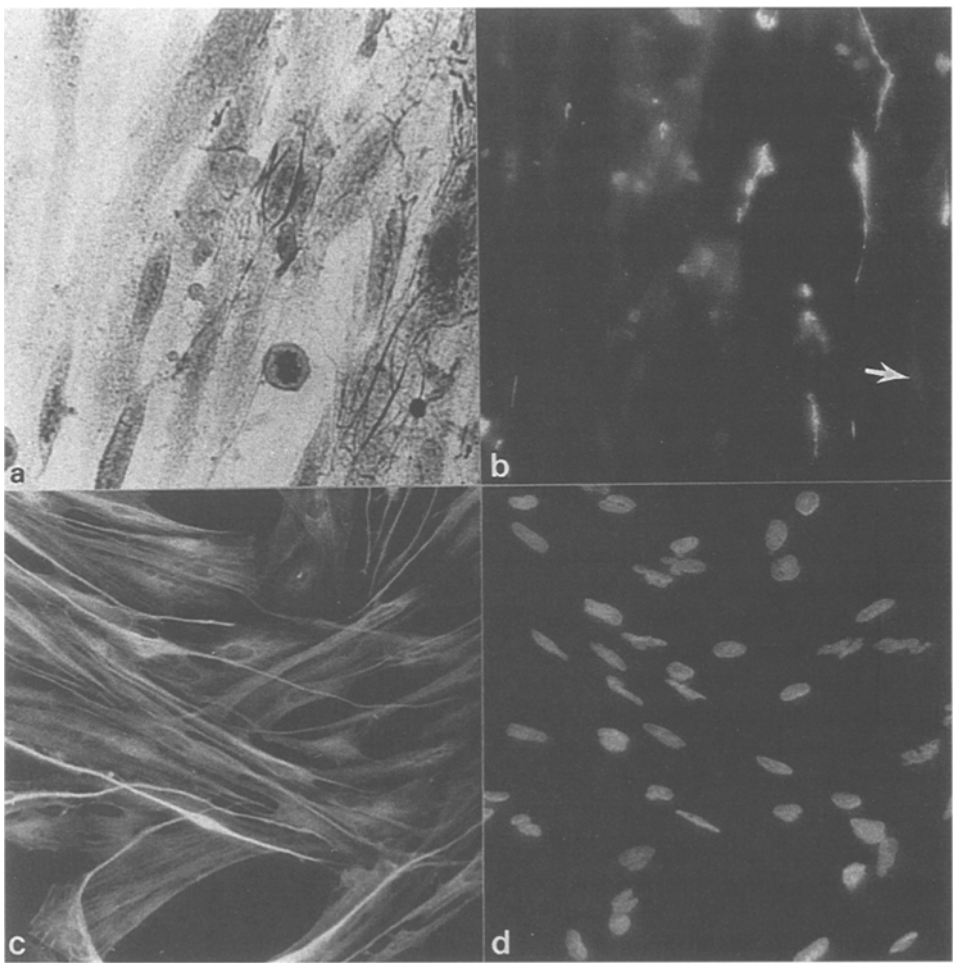
All four neoplasms were astrocytic gliomas of high grade malignancy. Three were glioblastoma multiforme, grade IV by World Health Organization (WHO) criteria of diagnosis and grade [24]. The other (glioma number 3) was an anaplastic astrocytoma, grade III. By new WHO criteria this third glioma would also be considered a glioblastoma [5].

The following features were evident in the tissues of all gliomas studied. ECM components (reticulin,

Table 3. Changes in extracellular matrix, endothelial, and glial components of individual gliomas upon cultivation *in vitro*

Glioma number & diagnosis	Ratio of cultivated cells to cells *in situ*[a]					
	Reticulin	Type IV collagen	Laminin	Fibronectin	*Ulex europaeus* ligand	GFAP
1 – Glioblastoma	2.7	1.6	2.3	4.5	0	0.12
2 – Glioblastoma	3.1	3.5	2.3	4.0	0	0.16
3 – Anaplastic astrocytoma	2.8	3.0	1.6	3.0	0	0
4 – Glioblastoma	1.3	1.8	0.28	0.17	0	1.2

[a] For each of the six components, this is the percentage of positive cells *in vitro* divided by the percentage of positive cells *in situ*.



*Figs 4–5.*Features of glioma number 4 that represent a unique pattern of gene product expression in cultured cells.
Fig. 4. Reticulin (A) and collagen type IV (B) are both expressed. Intracellular pro-collagen (arrow) was used for quantitation rather than the brighter extracellular collagen. Unlike the other gliomas, many cells express GFAP (C), their nuclei counterstained with DAPI (D) (original magnification: A, × 500; B, × 665; C and D, × 275). Tissue (not illustrated) was similar to tissue in Figs 1–3.

type IV collagen, laminin, and fibronectin) localized within the walls of abnormal vascular proliferations, but not in parenchyma of glioma tissue excised *in situ* (Figs 1 and 2, Table 1). *Ulex europaeus* bound endothelial cells (Fig. 3A); cells binding this marker were uniformly lost upon cultivation *in vitro* (Table 2 and Fig. 3B).

Changes in percentages of each marker that occurred during *in vitro* cultivation were calculated as the numerical ratio of percentage of positive cells *in vitro* divided by the percentage of positive cells *in situ* (Table 3). Three of these gliomas expressed each of the four extra-endothelial vascular ECM components on a greater percentage of cells *in vitro* than *in situ* (Figs 1 and 2, Table 3). The average increase in percentages of cells with reticulin, collagen, laminin, or fibronectin was between two-fold and four-fold. These same three gliomas retained few cells expressing GFAP upon cultivation (Tables 2 and 3). Gliomas 1 and 2 reevaluated in their tenth passage had lost all GFAP and retained all four ECM markers.

One glioma (number 4) produced a pattern of antigenic changes different than the other gliomas. Its retention of both parenchymal and specific vascular markers was striking. It retained GFAP and reticulin-positive cells, and it retained a smaller percentage of cells with fibronectin and laminin (Fig. 4, Ta-

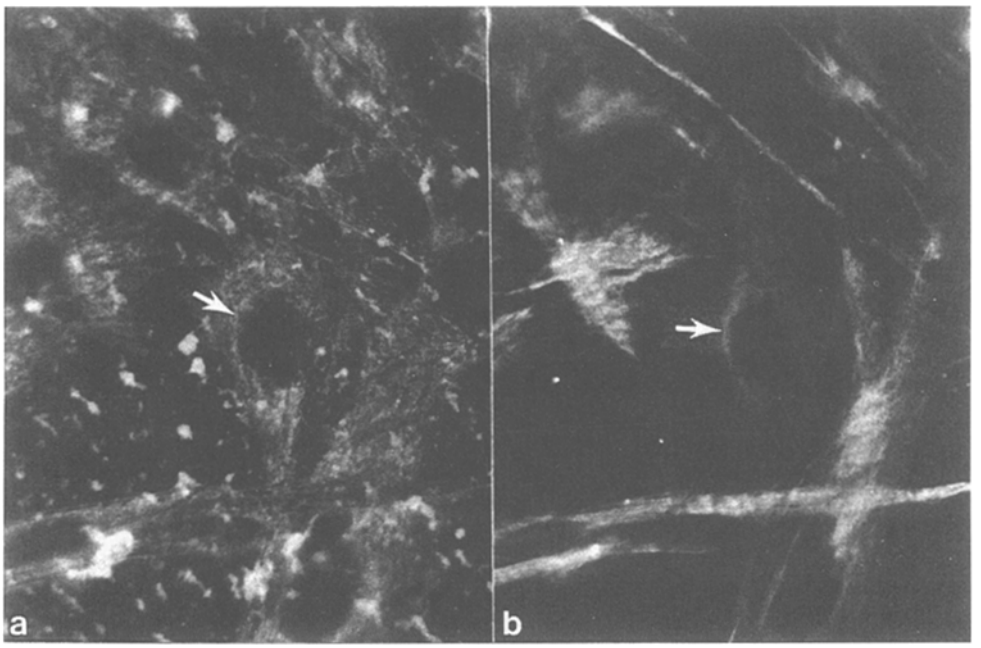


Fig. 5. Double labeling immunofluorescence for fibronectin (A) and GFAP (B) of cells cloned from glioma number 4. Cells express both markers in their cytoplasm surrounding shadows of the same negative nucleus (arrows).

ble 3). Type IV collagen-positive cells increased. To determine whether multiple cell populations might explain the mixture of vascular and parenchymal markers expressed by this glioma, it was cloned at limiting dilution. Clones expressed the markers of the parent line, but tended to show more ECM expression. Double labeling confirmed expression of vascular ECM and parenchymal markers in the same cell (Fig. 5).

To determine which, if any, of these cells cultured from gliomas were neoplastic, *in situ* hybridization (ISH) for pericentromeric alpha satellite DNA sequences of chromosomes 7 and 10 was applied to fixed cell cultures. Gliomas frequently have more than the normal pair of chromosomes 7, and malignant gliomas often lose all or a part of one chromosome 10 [45]. Results indicate a profile of ISH signals typical of a malignant glioma in cells cultured from glioma 4 (Table 4). Cells cultured from glioma 2 gave predominantly diploid ISH signal profiles similar to non-neoplastic control cells. Remarkably, cells from glioma 1 gave a bizarre pattern of aneuploid signals (Table 4). While clearly neoplastic, this pattern was not typical of a glioma.

Table 4. Hybridization signals of chromosomal satellite DNA of cells cultured *in vitro* from gliomas and control fibroblasts

Source of cells	Chromosome number	Percentage of fluorescent *in situ* hybridization signals						
		1	2	3	4	5	6	>6
Glioma 1	7	42	39	12	4	2	1	1
	10	11	12	15	16	13	11	22
Glioma 2	7	52	46	1	0.3			
	10	26	70	4	0.4			
Glioma 4	7	16	21	38	18	5	2	0.5
	10	73	18	6	2	0.5		
Fibroblasts	7	29	67	5				
	10	11	89					

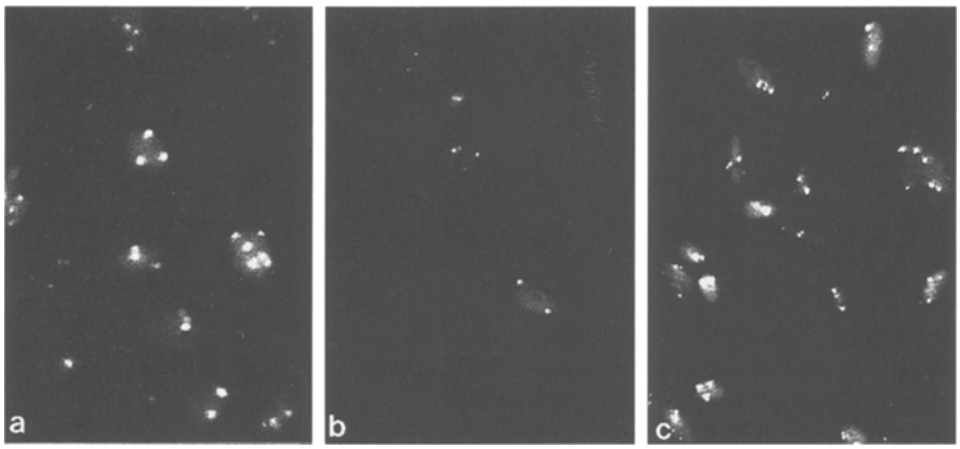


Fig. 6. Fluorescent *in situ* hybridization signals for chromosome 7 in cultured cells of glioma 1 (A), glioma 2 (B), and glioma 4 (C) (original magnifications × 450).

Discussion

All tumors, when studied immediately after biopsy, had essentially the same staining pattern, and the results of this study consisted of *in vitro* changes only. All ECM components of gliomas 1–3 increased upon culture. Some more than tripled their original percentages of positive cells in tissue. The gliomas that tended to lose GFAP upon cultivation generated a consistent direction of change toward the precise phenotype of non-endothelial cells from the walls of glioma vessels. This occurred in three gliomas among the four ECM components, GFAP and one lectin marker examined, a total of 18 individual observations. With each observation allowing change in the same or opposite direction as its counterpart, the possibility that this consistent pattern of phenotypic conversion resulted from random phenomena is less than 0.1%. The similarity of changes among these three cases provides evidence that they represent the most common form of transformation of ECM of gliomas. These results are most compatible with the possibility that mesenchymal cells in walls of their abnormal vascular proliferations outgrew the other cells cultured from these three gliomas. This would explain the loss of epidermal growth factor receptor gene amplification during glioma culture observed in other studies, since this gene is amplified in glioblastoma parenchyma rather than vascular proliferations [23, 46–48]. Interphase cytogenetic analysis indicates that these cells have either normal or neoplastic chromosomal signals.

While the possibility of cellular overgrowth producing changes in glioma phenotype has been previously proposed on the basis of descriptive evidence [15, 22, 49, 50] there have been minimal quantitative data to support this proposal. Many glioma cells in culture contain fibronectin, reticulin, type IV collagen, and laminin [16, 28, 29, 51, 52]. This contrasts with other studies that show relatively focal representation of these ECM components in glioma tissues [4, 6–9]. Despite this suggestion of altered ECM expression from different studies, only fibronectin has been documented to increase on a cell percentage basis upon culture of individual gliomas [51]. The present study investigated all of these ECM components on a cell percentage basis in tissue and culture to determine whether such changes were occurring and their extent.

Highly intriguing is the single glioma in this study that, upon cultivation, did not convert to a cellular phenotype encountered in its tissue. A mixture of cells of different lineage does not explain this phenotype, since individual cells with markers of both glial and mesenchymal lineage were found after cloning and double labeling. There are more than one possible explanation for these changes resulting in the special array of gene products expressed by this glioma culture:

1. It may represent outgrowth of a rare type of glio-

ma cell 'frozen' between expression of glial and expression of mesenchymal features. However, no cell of this phenotype was found in the tissue of this glioma.

2. Glioma parenchymal cells and cells within vascular proliferations may each produce an ECM. Glioma cells might then deposit this ECM at the vessel, possibly via their astrocytic foot processes that extend to meet the vascular adventitia. These foot processes might be analogous to the basal layer of an epithelial cell but located many microns away from their cell body. If this were the case, the ECM in this culture could originate in glioma parenchymal cells.
3. The components could be subject to complex alterations of gene expression which for unknown reasons favor expression of mesenchymal and/or vascular ECM antigens, but also allow continued expression of glial antigen.

Intracellular GFAP can be modified to a degree in certain gliomas by changes in hormones, cellular density, configuration, or growth substrate [53–59]. Since production of ECM proteins by glioma cultures has been recognized [60], investigations of modulation of production of these ECM components have begun. Butyrate induces fibronectin and collagen gene expression in one established glioma cell line [61]. Of particular interest is the association of a human immunodeficiency virus regulatory protein with increased transcription of fibronectin and alpha I type I collagen promoters [62]. These studies pertain to the present observations by demonstrating that some coordinated expression of ECM components can be stimulated by exogenous agents. In the future, it would be interesting to determine whether these, or other agents, could produce the transformation encountered in this fourth glioma in the present study.

These questions pertaining to mesenchymal drift are fundamental to a better understanding of gliomas which could provide therapeutic insights. For example, monoclonal antibody therapies targeting gliomas have had limited success, perhaps because they have aimed at a moving target of changing gene expression. If these changes are a manifestation of unlimited ability of the glioma cell to alter its gene expression in response to the effects of exogenous agents, then the potential of glioma cells to escape therapy aimed at one phenotype by repeated phenotypic alterations is a serious concern. Changes that result from a limited number of different cell populations in gliomas, one of which outgrows the other, are more likely to be susceptible to targeted therapy like immunotherapy or growth-factor biomodulation, since each cell population can be individually managed.

Acknowledgements

Mr. Phu K. Nguyen, Ms. R. Lynn Castle and Ms. Mary Laug carefully stained some of the preparations. Members of the Section of Neurosurgery, Department of Surgery, University of Michigan Medical School, made valuable contributions to the study. Dr. Hynda K. Kleinman and Dr. George R. Martin, National Institute of Dental Research, generously donated the antiserum to type IV collagen. Ms. Peggy Otto, Ms. Shannon Grace, Ms. Charlene Fegan and Ms. Denise Lindsay skillfully prepared the manuscript. Dr. McKeever is supported by National Cancer Institute Grants CA47558 and CA54104. This work was supported by National Cancer Institute Grant CA47558, National Institutes of Health, U.S. Public Health Service, awarded to Dr. McKeever.

References

1. Paetau A: Glial fibrillary acidic protein, vimentin and fibronectin in primary cultures of human glioma and fetal brain. Acta Neuropathol (Berl) 75: 448–455, 1988
2. McKeever PE: Human astrocytic neoplasms. In: Murphy S (ed) Astrocytes: Pharmacology and Function. Academic Press, New York 399–436, 1993
3. Schmitt HP: Rapid anaplastic transformation in gliomas of adulthood, 'Selection' in neuro-oncogenesis. Pathol Res Prac 176: 313–323, 1983
4. Chronwall BM, McKeever PE, Kornblith PL: Glial and non-glial neoplasms evaluated on frozen section by double immunofluorescence for fibronectin and glial fibrillary acidic protein. Acta Neuropathol (Berl) 59: 283–287, 1983
5. Kleihues P, Burger PC, Scheithauer BW: The new WHO classification of brain tumours. Brain Pathology 3: 255–268, 1993

6. Bellon G, Caulet T, Cam Y, Pluot M, Poulin G, Pytlinska M, Bernard MH: Immunohistochemical localization of macromolecules of the basement membrane and extracellular matrix of human gliomas and meningiomas. Acta Neuropathol (Berl) 66: 245–252, 1985
7. McComb RD, Bigner DD: Immunolocalization of laminin in neoplasms of the central and peripheral nervous systems. J Neuropathol Exp Neurol 44: 242–253, 1985
8. McComb RD, Moul JM, Bigner DD: Distribution of type IV collagen in human gliomas: comparison with fibronectin and glioma-mesenchymal matrix glycoprotein. J Neuropathol Exp Neurol 46: 623–633, 1987
9. McComb RD, Bigner DD: Immunolocalization of monoclonal antibody-defined extracellular matrix antigens in human brain tumors. J Neuro-Oncol 3: 181–186, 1985
10. Jacque CM, Kujas M, Poreau A, Raoul M, Collier P, Racadot J, Baumann N: GFA and S-100 protein levels as an index for malignancy in human gliomas and neurinomas. J Natl Cancer Inst 62: 479–483, 1979
11. Schiffer D, Giordana MT, Mauro A, Migheli A: GFAP, FVIII/RAg, laminin, and fibronectin in gliosarcomas: An immunohistochemical study. Acta Neuropathol 63: 108–112, 1984
12. Weaver D, Vandenberg S, Park TS, Jane JA: Selective peripancreatic sarcoma metastases from primary gliosarcoma. J Neurosurg 61: 599–601, 1984
13. McKeever PE, Wichman A, Chronwall BM, Thomas C, Howard R: Sarcoma arising from a gliosarcoma. So Med J 77: 1027–1032, 1984
14. Jones TR, Ruoslahti E, Schold SC, Bigner DD: Fibronectin and glial fibrillary acidic protein expression in normal human brain and anaplastic human gliomas. Cancer Res 42: 168–177, 1982
15. Bilzer T, Stavrou D, Wechsler W, Wohler B, Keiditsch E: Antigen variation in a human glioblastoma: from the primary tumor to the second recurrence, permanent cell line and xenotransplantation tumors. Anticancer Res 11: 547–553, 1991
16. McKeever PE, Fligiel SEG, Varani J, Hudson JL, Smith D, Castle RL, McCoy P: Products of cells cultured from gliomas: IV. Extracellular matrix proteins of gliomas. Int J Cancer 37: 867–874, 1986
17. McKeever PE, Hood TW, Varani J, Taren JA, Beierwaltes WH, Wahl R, Liebert M, Nguyen PK: Products of cells cultured from gliomas: V. Cytology and morphometry of two cell types cultured from gliomas. J Natl Cancer Inst 78: 75–84, 1987
18. McKeever PE, Smith BH, Taren JA, Wahl RL, Kornblith PL, Chronwall BM: Products of cells cultured from gliomas. VI. Immunofluorescent, morphometric, and ultrastructural characterization of two different cell types growing from explants of human gliomas. Am J Pathol 127: 358–372, 1987
19. Rutka JT, Giblin JR, Apodaca G, De Armond SJ, Stern R, Rosenblum ML: Inhibition of growth and induction of differentiation in malignant human glioma cell line by normal leptomeningeal extracellular matrix proteins. Cancer Res 47: 3515–3522, 1987
20. Wikstrand CJ, Bigner SH, Bigner DD: Demonstration of complex antigenic heterogeneity in a human glioma cell line and eight derived clones by specific monoclonal antibodies. Cancer Res 43: 3327–3334, 1983
21. Bongcam-Rudloff E, Nister M, Betsholtz C, Wang JL, Stenman G, Huebner K, Croce CM, Westermark B: Human glial fibrillary acidic protein: Complementary DNA cloning, chromosome localization, and messenger RNA expression in human glioma cell lines of various phenotypes. Cancer Res 51: 1553–1560, 1991
22. Franks AJ, Burrow HM: *In vitro* heterogeneity in human gliomas. Are all transformed cells of glial origin? Anticancer Res 6: 625–630, 1986
23. Stefanik DF, Rizkalla LR, Soi A, Goldblatt SA, Rizkalla WM: Acidic and basic fibroblast growth factors are present in glioblastoma multiforme. Cancer Res 51: 5760–5765, 1991
24. Zulch KJ: Histological Typing of Tumours of the Central Nervous System. International Histological Classification of Tumours, no. 21. World Health Organization, Geneva 1–66, 1979
25. Kornblith PL: Role of tissue culture in prediction of malignancy. Clin Neurosurg 23: 346–361, 1978
26. Black PM, Kornblith PL, Davison PF, Liszczak TM, Merk LP, Smith BH, McKeever PE, Quindlen EA: Immunological, biochemical ultrastructural, and electrophysiological characteristics of a human glioblastoma-derived cell culture line. J Neurosurg 56: 62–72, 1982
27. McKeever PE, Quindlen E, Banks MA, Williams U, Kornblith PL, Laverson S, Greenwood MA, Smith B: Biosynthesized products of cultured neuroglial cells: I. Selective release of proteins by cells from human astrocytomas. Neurol 31: 1445–1452, 1981
28. Ponten J, Westermark B: Properties of human malignant glioma cells *in vitro*. Med Biol 56: 184–193, 1978
29. McKeever PE, Fligiel SEG, Varani J, Castle RL, Hood TW: Products of cells cultured from gliomas: VII. Extracellular matrix proteins of gliomas which contain glial fibrillary acidic protein. Lab Invest 60: 286–295, 1989
30. Peterkofsky B: The effect of ascorbic acid on collagen polypeptide synthesis and proline hydroxylation during the growth of cultured fibroblasts. Arch Biochem Biophys 152: 318–328, 1972
31. Baldwin JR, McKeever PE, Booker TR: Products of cultured neuroglial cells. II. The production of fibronectin by C6 glioma cells. Neurochem Res 10: 601–610, 1985
32. Berman J, Stoner G, Dawe C, Rice J, Kingsbury E: Histochemical demonstration of collagen fibers in ascorbic-acid-fed cell cultures. In Vitro 14: 675–685, 1978
33. Kalimo H, Lehto M, Nanto-Salonen K, Jalkanen M, Risteli L, Risteli J, Narva EV: Characterization of perivascular reticulin network in a case of primary brain lymphoma. Immunohistochemical demonstration of collagen types I, III, IV and V; laminin; and fibronectin. Acta Neuropathol (Berl) 66: 299–305, 1985
34. Karttunen T, Alavaikko M, Apaja-Sarkkinen M, Autio-Harmainen H: An immunohistochemical study of laminin, type-IV collagen and type-III pN-collagen with relation to reticular fibers in Hodgkin's disease. Int J Cancer 41: 52–58, 1988

35. Foidart JM, Berman JJ, Paglia L, Rennard SI, Abe S, Peratoni A, Martin GR: Synthesis of fibronectin, laminin, and several collagens by a liver-derived epithelial cell line. Lab Invest 42: 525–532, 1980
36. Rennard SI, Bere R, Martin GR, Foidart JM, Gehran RP: Enzyme-linked immunoassay (ELISA) for connective tissue components. Anal Biochem 104: 205–214, 1980
37. Mukhopadhyay SK, McKeever PE, Greenberg HS, Junck L, Chandler WF, Ensminger W: Random sampling by glitter drop method. Life Sci 46: 507–512, 1990
38. Matthews DE, Farewell VT: Using and Understanding Medical Statistics, 2nd ed. Karger, New York 1–288, 1988
39. Lichter P, Cremer T, Borden J, Manuelidis L, Ward DC: Delineation of individual human chromosomes in metaphase and interphase cells by *in situ* suppression hybridization using recombinant DNA libraries. Hum Genet 80: 224–234, 1988
40. Pinkel D, Landegent J, Collins C, Fuscoe J, Segraves R, Lucas J, Gray J: Fluorescence *in situ* hybridization with human chromosome-specific libraries: Detection of trisomy 21 and translocations of chromosome 4. Proc Natl Acad Sci USA 85: 9138–9142, 1988
41. Kim SY, Lee JS, Ro JY, Gay ML, Hong WK, Hittelman WN: Interphase cytogenetics in paraffin sections of lung tumors by non-isotopic *in situ* hybridization: Mapping genotype/phenotype heterogeneity. Am J Pathol 142: 307–317, 1993
42. McKeever PE, Balentine JD: Histochemistry of the nervous system. In: Spicer SS (ed) Histochemistry in Pathologic Diagnosis. Marcel Dekker, New York, pp 871–957, 1987
43. McKeever PE, Laverson S, Oldfield EH, Smith BH, Gadille D, Chandler WF: Stromal and nuclear markers for rapid identification and pituitary adenomas at biopsy. Arch Pathol Lab Med 109: 509–514, 1985
44. Arnoldus EP, Wolters LB, Voormolen JH, Van Duinen SG, Raap AK, Van der Ploeg M, Peters AC: Interphase cytogenetics: A new tool for the study of genetic changes in brain tumors. J Neurosurg 76: 997–1003, 1992
45. Bigner SH, Mark J, Bullard DE, Mahaley MS, Bigner DD: Chromosomal evolution in malignant human gliomas starts with specific and usually numerical deviations. Cancer Genet Cytogenet 22: 121–135, 1986
46. Hermansson M, Nister M, Betsholtz C, Helden CH, Westermark B, Funa K: Endothelial cell hyperplasia in human glioblastoma: coexpression of mRNA for platelet-derived growth factor (PDGF) B chain and PDGF receptor suggests autocrine growth stimulation. Proc Natl Acad Sci USA 85: 7748–7752, 1988
47. Bigner SH, Humphrey PA, Wong AJ, Vogelstein B, Mark J, Friedman HS, Bigner DD: Characterization of the epidermal growth factor receptor in human glioma cell lines and xenografts. Cancer Res 50: 8017–8022, 1990
48. Westphal M, Brunken M, Rohde E, Herrmann HD: Growth factors in cultured human glioma cells: Differential effects of FGF, EGF and PDGF. Cancer Lett 38: 283–296, 1988
49. Haynes LW, Davis BE, Mitchell J, Weller RO: Cell proliferation in explant cultures of malignant gliomas. Acta Neuropathol (Berl) 42: 87–90, 1978
50. Sipe JC, Herman MM, Rubinstein LJ. Electron microscopic observations on human glioblastomas and astrocytomas maintained in organ culture systems. Am J Pathol 73: 589–606, 1973
51. McKeever PE, Chronwall BM: Early switch in glial protein and fibronectin markers on cells during the culture of human gliomas. Ann NY Acad Sci 435: 457–459, 1985
52. Bigner DD, Bigner SH, Ponten J, Westermark B, Mahaley MS, Ruoslahti E, Herschman H, Eng LF, Wikstrand CJ: Heterogeneity of genotypic and phenotypic characteristics of fifteen permanent cell lines derived from human glioma. J Neuropathol Exp Neurol 40: 210–229, 1981
53. Bissell MG, Eng LF, Herman MM, Bensch KG, Miles LEM: Quantitative increase of neuroglia-specific GFA protein in rat C-6 glioma cells *in vitro*. Nature 255: 633–634, 1975
54. Labourdette G, Mahony JB, Brown IR, Marks A: Regulation of synthesis of a brain-specific protein in monolayer cultures of clonal glial cells. Eur J Biochem 81: 591–597, 1977
55. Rutka JT: Effects of extracellular matrix proteins on the growth and differentiation of an anaplastic glioma cell line. Can J Neurol Sci 13: 301–306, 1986
56. Korosue K, Takeshita I, Mannoji H, Fukui M: Interferon effects on multiplication, cytoplasmic protein and GFAP content, and morphology in human glioma cells. J Neuro-Oncol 1: 69–76, 1983
57. Rutka JT, De Armond SJ, Giblin J, McCullock JR, Wilson CB, Rosenblum ML: Effects of retinoids on the proliferation, morphology and expression of glial fibrillary acidic protein of an anaplastic astrocytoma cell line. Int J Cancer 42: 419–427, 1988
58. Shafit-Zagardo B, Kume-Iwaki A, Goldman JE: Astrocytes regulate GFAP mRNA levels by cyclic AMP and protein kinase C-dependent mechanisms. Glia 1: 346–354, 1988
59. Weir MD, Thomas DGT: Effect of dexamethasone on glutamine synthetase and glial fibrillary acidic protein in normal and transformed astrocytes. Clin Neuropharm 7: 303–306, 1984
60. Alitalo K, Bornstein P, Vaheri A, Sage H: Biosynthesis of an unusual collagen type by human astrocytoma cells *in vitro*. J Biol Chem 258: 2653–2661, 1983
61. Tang ST, Ko LW, Lee YH, Wang FF: Induction of fos and sis proto-oncogenes and genes of the extracellular matrix proteins during butyrate induced glioma differentiation. Biochim Biophys Acta 1048: 59–65, 1990
62. Taylor JP, Cupp C, Diaz A, Chowdhury M, Khalili K, Jimenez SA, Amini S: Activation of expression of genes coding for extracellular matrix proteins in Tat-producing glioblastoma cells. Proc Natl Acad Sci USA 89: 9617–9621, 1992

Address for offprints: P.E. McKeever, Department of Pathology, Box 0602, M3218 Medical Science Building 1, The University of Michigan Medical School, 1301 Catherine Road, Ann Arbor, Michigan 48109-0602, USA