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Products of Cells Cultured From Gliomas

VI. Immunofluorescent, Morphometric, and Ultrastructural Characterization of Two Different Cell Types Growing From Explants of Human Gliomas

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Explants derived from human gliomas have been characterized with respect to their cellular outgrowth pattern after 1–22 weeks in culture. A mat of cells which were fibronectin (FN)-positive and glial fibrillary acidic protein (GFAP)-negative (hereafter designated FN$^+$ cells) with a polygonal, flat morphology covered the growth substrate in a swirling pattern for a mean diameter of 9.2 mm around FN$^+$ explants. FN$^+$ cells showed ruffled plasmalemma, dilated rough endoplasmic reticulin (RER), and extracellular filamentous strands. Rare desmosomes were compatible with at most minor leptomeningeal components or differentiation. FN$^+$ cells predominated in six of seven cultures at passage 2, and their features were the same from various high-grade gliomas and gliosarcoma. Around other explants, elongated or stellate cells which were GFAP$^+$ and FN$^-$ grew in a netlike pattern with little cell-to-cell contact. These GFAP$^+$ cells surrounded explants at a mean diameter of 2 mm, substantially less than FN$^+$ cells ($P < 0.005$), and they grew more slowly than FN$^+$ cells around explants. GFAP$^+$ cells had an area/perimeter ratio which was less than that of FN$^+$ cells. GFAP$^+$ cells contained abundant intracellular filaments, rare desmosomes, and narrow RER cisternae. In mixed explants, GFAP$^+$ cells often grew on top of FN$^+$ cells. Individual cells which stained for both GFAP and FN were evident only from one glioma (8% doubly positive). Cells negative for both proteins resembled FN$^+$ cells morphologically. Frozen sections of original glioma tissue showed FN$^+$ vessel walls and GFAP$^+$ parenchyma. Results are evidence for very early overgrowth of a preexistent FN$^+$ cell type distinct from the GFAP$^+$ parenchymal cell. The features of this distinct cell type are mesenchymal and resemble the proliferating vascular elements of gliomas *in situ*. The tendency for GFAP$^+$ cells to grow on top of these FN$^+$ cells suggests a feeder layer interaction. More knowledge of the origins and interactions of these two cell types may increase our understanding of the mechanism of antigenic changes in gliomas and may provide clues to improved therapeutic approaches. (Am J Pathol 1987, 127:358–372)

AS GLIOMAS grow *in situ* within patients, they become more resistant to therapy, and some of them become more malignant. Increased malignancy in gliomas is associated with decreased expression of an antigen which is characteristic of normal, well-differentiated glia, glial fibrillary acidic protein (GFAP), and increased expression of fibronectin (FN).[1–5]

During explantation, glioma cells decrease expression of GFAP and increase expression of FN.[6,7] Al-

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though the antigenic instabilities are similar to those observed during tumor progression *in situ*, during explantation the changes in antigens occur more completely and over a shorter duration of time than *in situ*, days as compared with months.[1-7] The majority of neoplastic established glioma cell lines resemble cells obtained from gliomas after explantation in that they express more fibronectin than GFAP.[7-10]

It is important to understand the mechanism of this ability of glioma cells to switch their antigens. If this switch is a manifestation of the ability of the glioma cell to alter its phenotypic expression of its genotype in response to stress, then the potential of glioma cells to escape immunotherapy by repeatedly changing their antigens is a serious concern. If, on the other hand, this switch is the consequence of different and antigenically stable cell populations in gliomas, one of which outgrows the other, then it is more likely that gliomas will be susceptible to immunotherapy, because both cell populations could be targeted by the immunotherapeutic approach.

In order to better understand this switch in glioma antigens, we examined the point at which this switch was occurring, the explanation stage (passage zero) of culture. The results are evidence for the presence of different populations of cells, since antigenic, morphometric, and ultrastructural features cluster into two distinct groups among the cells positive for the markers employed. A portion of these results has been reported in an abstract.[11]

Materials and Methods

Explants

Explant cultures of high grade gliomas were prepared as described.[12-14] The original diagnosis of each astrocytoma (Grade III, Grade IV, or glioblastoma multiforme) was retained for accuracy, even though this resulted in some overlap of terminology. Under a vertical laminar flow hood, the necrotic areas, macroscopic blood vessels, and any encapsulating material were cut away so that only the most homogeneous, viable tumor tissue was used for culture. This remaining tissue was then cut into approximately 1-mm pieces by means of two scalpels in a scissorlike motion.

The 1-mm pieces of tumor were placed in either a 25-sq cm or 75-sq cm tissue culture flask. To ensure adhesion of the explants, we then inverted the flask and placed it in a well-humidified incubator at 37 C. After 30 minutes, enough Ham's F10 medium with 10% fetal calf serum was added to the flask that the explants were barely covered. On the next day, more medium was added (3 ml/25-sq cm flask). The flasks were put back into the incubator and not disturbed for 1 week. Cases 1–4 were grown on glass microscopic slides in Petri dishes, which were never inverted. Twelve cases were intended for study as explants with subsequent cases to be studied after passages in culture.

Immunofluorescent Staining

Specimens were fixed in 4% formaldehyde in 0.02 M sodium phosphate buffer, pH 7.2 (PBS), for 1 minute, triple-rinsed in PBS, and permeabilized with 0.3% Saponin in Dulbecco's PBS with 1 mM EGTA for 15 minutes. After triple PBS rinsing, they were incubated for 10 minutes with fluorescein-conjugated IgG fraction of anti-FN (Cappel) diluted 1 : 50 with PBS after reconstitution, triple-rinsed in PBS, incubated for 10 minutes with rabbit anti-GFAP (kindly provided by Dr. Lawrence Eng, Stanford University)[15] diluted 1 : 100, triple-rinsed in PBS, and incubated for 5 minutes with rhodamine-conjugated goat anti-rabbit IgG (Cappel) diluted 1 : 50, rinsed in PBS, and mounted in glycerol. The entire procedure allowed simultaneous staining of a single specimen for both antigens. This procedure was used on both cultured cells and on frozen sections of the original glioma tissue. The result was viewed in a Zeiss ICM 405 fluorescence microscope with 440–490-nm excitation, 510-nm reflector, and LP520 plus KP560 barrier filters for fluorescein and 534–558-nm excitation and 580-nm reflector for rhodamine.

In control incubations where the anti-FN serum had been absorbed with human fibronectin (0.2 mg/ml) for 30 minutes, no staining of the sections was obtained. Controls included incubation with irrelevant antibodies, such as fluorescein-labeled anti-Con A, used in place of anti-FN. Antibody deletion controls replaced the primary anti-GFAP antiserum with normal rabbit serum. All of these controls confirmed the specificity of the stains.

Image Analysis

Micrographs of the same field were taken under fluorescein and rhodamine excitation, phase-contrast illumination, and scanning electron microscopy (SEM). For SEM, the $GFAP^+$ and FN^+ cells were relocated by their precise morphology and distribution. Morphometry on 50 cells of each phenotype was performed on a modified Bausch and Lomb Omnicon (FAS II) image processing system interfaced with a JEOL JSM 35C scanning electron microscope. The

system permits rapid (5–10-minute) collection of measurement parameters, including area and perimeter, in 100 or more cells.

Scanning Electron Microscopy

After fluorescence microphotography of fields to allow subsequent identification of FN^+ and $GFAP^+$ cells, the cells were postfixed for 15 minutes in cold 2% osmium tetroxide, rapidly dehydrated through chilled graded ethanols (1–2 minutes each) and immediately critical-point-dried with liquid CO_2. Specimens were gold-coated and examined with a JEOL JSM 35C scanning electron microscope.

Transmission Electron Microscopy

Following fluorescence microphotography of fields for identification of FN^+ and $GFAP^+$ cells, specimens were subsequently fixed in 3% formaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer for 1 hour osmicated for 2 hours, dehydrated through a series of graded methanols (5-minute changes) and three 10-minute changes of 100% methanol, and embedded in Poly-Bed 812.

The flasks and plastic in which the cultures were embedded were separated with dental forceps. Fields of previously photographed FN^+ and $GFAP^+$ cells were relocated in the resin stained with toluidine blue O. Sections of the tissue specimens cut 1 μ thick were restained with toluidine blue O for light microscopy. Thin sections, 60–70 nm thick, were counterstained for 5 minutes with a saturated solution of uranyl acetate and for 3 minutes with 0.5% lead citrate and examined in an electron microscope.

Results

FN^+ and $GFAP^+$ Cells in Explants

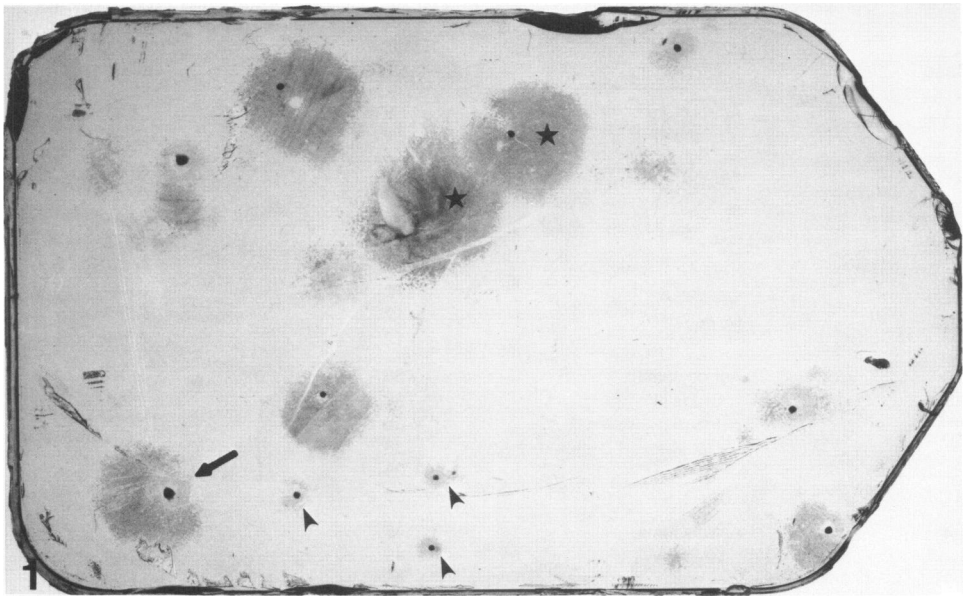
Two patterns of cellular outgrowths from explants occurred (Figure 1). In many explants first stained for FN and GFAP and then relocated after staining for light microscopy, different morphology of two populations of cells was evident (Figures 2 and 3). In the first pattern, cells which were positive for FN and negative for GFAP markers (hereafter designated FN^+ cells) predominated around some explants. Many of these cells were polygonal and flat and formed a continuous mat, which grew out from the explants several millimeters (Figure 3 and Table 1). FN^+ cells showed either none or short processes and were flatter than $GFAP^+$ cells (Figures 4–6) with ruffling of the plasmalemma. Unfortunately, morphologic criteria alone were insufficient to identify each cell as FN^+ or $GFAP^+$ because individual cells varied substantially from the general pattern of the population as a whole. Computerized image analysis of FN^+ cell populations showed relatively high area/perimeter ratios (Table 2). Two-thirds of the cases originally set aside for study as explants grew cells suitable for electron microscopy, quantitation of outgrowth, or morphometry.

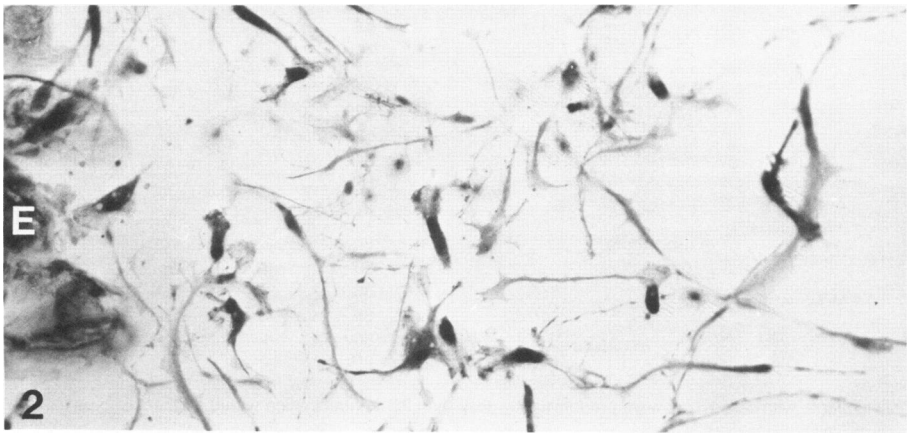
FN^+ cells were abundant far from the original explants (Figures 1 and 3 and Table 1). Closer to the original explant, FN^+ cells grew on top of one another. On some mixed explants, these FN^+ cells were the growth substrate for small clusters of $GFAP^+$ cells (Figures 7–9). The shapes of each of these two cell populations were retained in mixed explants.

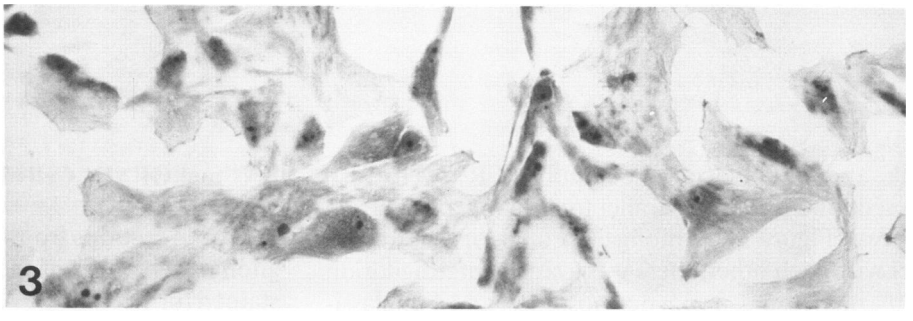
FN^+ cells contained abundant rough endoplasmic reticulum with dilated cisternae filled with amorphous material (Figure 10). Intermediate filaments of 8–9 nm and microtubules were sparse and located in the perinuclear cytoplasm in loose arrays. Filaments of 6–7 nm, resembling actin, were prominent on the cytoplasmic side of the plasmalemma which touched the culture surface. Among 90 profiles of cells in contact with each other, there were 6 interdigitations of plasmalemma, 2 spot desmosomes, and 7 junctions too poorly formed to be classified. Pinocytotic vesicles and vacuolar debris varied in amount among individual cells. On surfaces of FN^+ cells, extracellular filaments which lacked periodicity and varied in thickness reacted with immunoperoxidase stain for FN.

Not all of the cells which exhibited this morphology in culture were FN^+; some were negative for both FN and GFAP markers. These cells may represent a third subgroup identified with these two protein markers. However, definitive morphologic differences between the cells negative for both markers and the FN^+ cells were not found, and double-negative cells were usually in proximity to FN^+ cells. A higher percentage of the flat and polygonal cells from some gliomas expressed FN in regions crowded with these cells than at the outer perimeter, where cells with similar morphology did not touch one another.

Around other explants, a second pattern of outgrowth of cells which contained GFAP and lacked FN, hereafter called $GFAP^+$ cells, predominated (Figure 1). While many of these $GFAP^+$ cells contacted each other, the regions of cellular contact with other cells and with the culture flask were small compared with the regions of cellular contact of FN^+ cells (Figures 2, 4, and 8). The shape of many of the $GFAP^+$ cells was stellate because of radial extension of multiple processes. Clusters of most these cells resembled a handwoven fishnet. Other $GFAP^+$ cells had fewer







Figures 1–3—Explants were stained by double immunofluorescence for FN and GFAP. After the results of immunostaining were recorded, the entire flask was embedded in Epon and counterstained with toluidine blue. **Figure 1**—Explants of large diameter were positive for FN (*stars*), and explants of small diameter were positive for GFAP (*arrowheads*). This flask represents an unusually good separation of explants with $GFAP^+$ and FN^+ cells. A mixed explant (*arrow*) has $GFAP^+$ cells (*light area*) around the explant (*dark spot*) and FN^+ cells at the periphery. Actual size. **Figure 2**—Detail of an explant (*E*) marked with *arrowhead* in Figure 1 with outgrowth of $GFAP^+$ cells. (×200) **Figure 3**—Detail of FN^+ cells in the outer perimeter of an explant marked with a *star* in Figure 1. (Toluidine blue, ×200)

processes and were bipolar or unipolar (Figure 2), and a few were epithelioid, like FN^+ cells. The processes of many of the $GFAP^+$ cells appeared to be thinner than the processes of the FN^+ cells (Figure 7). The elongated, thin processes of the $GFAP^+$ cells were reflected in a mean area/perimeter ratio which was lower than the ratio for FN^+ cells (Table 2). These cells resembled the classic protoplasmic astrocyte in protoplasmic astrocytomas.

Surfaces of the $GFAP^+$ cells lacked the ultrastructural coating of extracellular filaments seen on the FN^+ cells and were quite smooth. The cellular processes were rounder than processes of FN^+ cells (Figure 4). Pseudopodia and circumferential swellings protruded from the cell bodies and the processes (Figure 11). The long, thin cellular processes were filled with compact bundles of 8–9-nm filaments oriented in parallel to the long axis of the process. Mitochon-

Table 1—Comparison of Diameters of Rings of FN+ and GFAP+ Cellular Outgrowths From Explants

Case	Diagnosis	Days in culture	Predominant cell type*	Diameter of ring of cells (mm)
1	Astrocytoma, Grade III	9	FN+	3.8
		9	GFAP+	0.8
2	Astrocytoma, Grade IV	12	GFAP+	1.6
		12	GFAP+	2.2
		12	GFAP+	2.0
		12	GFAP+	1.2
		12	GFAP+	2.0
		12	GFAP+	2.1
		12	GFAP+	3.1
		12	GFAP+	1.7
3	Astrocytoma, Grade III	38	FN+	13
		38	FN+	4.4
		38	FN+	3.3
		38	FN+	9.5
		38	FN+	6.5
		38	GFAP+	1.3
4	Astrocytoma, Grade IV	45	FN+	7.2
		45	FN+	6.0
		45	FN+	6.8
		45	FN+	17
		45	GFAP+	0.7
		45	GFAP+	0.5
5	Glioblastoma Multiforme	57	FN+	18
		57	FN+	21
		57	FN+	16
		57	FN+	5.5
		57	FN+	4.8
		57	FN+	5.1
6	Astrocytoma, Grade III	80	FN+	18
		80	FN+	15
		80	GFAP+	3.9
		80	GFAP+	2.2
		80	GFAP+	3.3
		80	GFAP+	3.2

*Only explants with cells which were predominantly (less than 2% contamination with the other cell type) either FN+ or GFAP+ were quantitated.

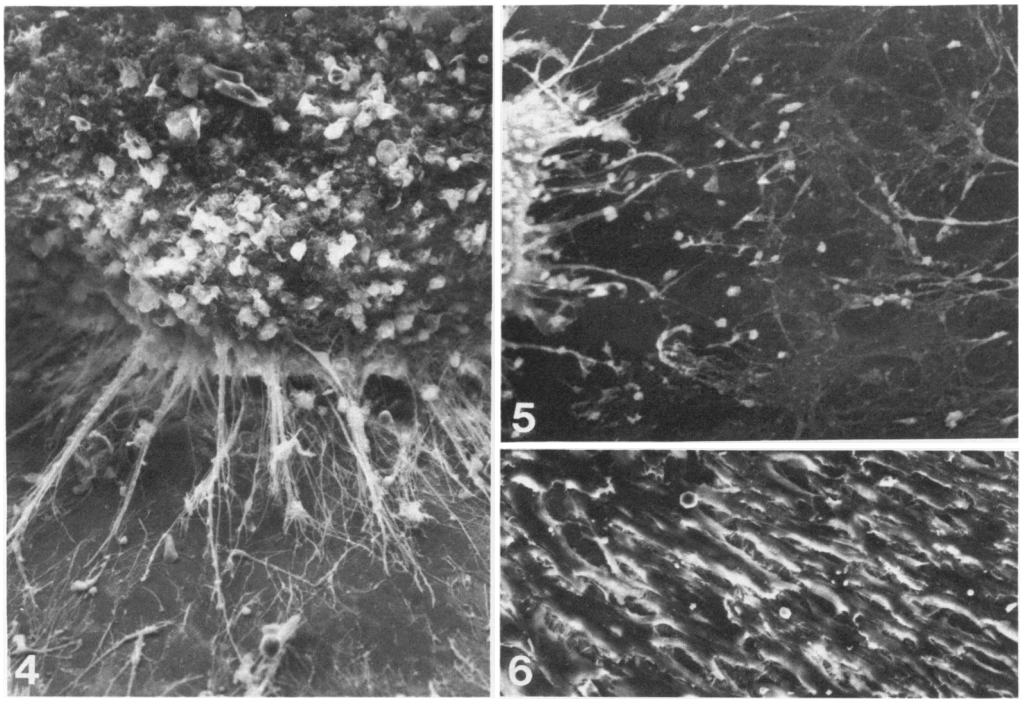
dria and endoplasmic reticulum (ER) were also present and oriented parallel to the long axis of each process (Figure 11). Among 66 profiles of cells in contact with each other, there was a single interdigitation of plasmalemma, 3 spot desmosomes, and 4 unclassifiable junctions.

The perikaryon of GFAP+ cells contained 8–9-nm filaments in bundles, mitochondria, and clear vacuoles up to 1 μ in diameter and bound by a single membrane. These vacuoles resembled dilated smooth ER or Golgi. Rough ER had narrow cisternae (Figure 11). There were few pinocytotic vesicles. Nuclei contained much euchromatin and heterochromatin marginated against the nuclear membrane. Some nuclei had multiple profiles of nucleoli with irregular borders.

FN+ and GFAP+ Cells in Tissue

Frozen sections from *in situ* gliomas, including the gliomas which provided the explants studied, were stained for FN and GFAP. GFAP+ cells which lacked FN composed the parenchyma of glioma tissue. Cellular processes resembling profiles of the processes extended from GFAP+ cells around explants were intertwined into a mat (Figure 12). The ultrastructure of these processes resembled the ultrastructure of processes of GFAP+ cells around explants in their content and orientation of 8–9-nm filaments, and other cytoplasmic structures.

FN+ regions were in vessels in the formations commonly referred to as vascular and endothelial proliferations (Figure 13), as judged by comparison of im-



Figures 4–6—Scanning electron-microscopic preparations of mixed explants. **Figure 4**—Processes of GFAP$^+$ cells are radiating out from the explant. (×220) **Figure 5**—Further from the explant, FN$^+$ cells form a mat on the surface. (×80) **Figure 6**—Far from the explant the FN$^+$ cells remain confluent. (×150)

munostained sections with phase microscopy and with serial hematoxylin and eosin (H&E)-stained sections. Most of the FN$^+$ reactivity was confined to these regions except in gliomas with meningeal invasion and a gliosarcoma. Gliomas with meningeal invasion and gliosarcomas were not among those studied as explants or at passage 2.

Cells Positive for Both FN and GFAP

The extensive overlapping of cells in glioma tissue and inability to define individual cell boundaries in tissue sections precluded identification of dual-labeled cells in glioma tissue. The interpretation of clusters of overlapping cells in explants was also difficult, but appeared to show cells positive for both GFAP and FN (Figures 14 and 15) of one glioma (Case 6). Light- and fluorescence-microscopic features of these cells were closer to the general features of most GFAP$^+$ cells than to those of FN$^+$ cells. FN on these cells was uniformly minimal and present as extracellular streaks (Figure 15). Ultrastructural features could not be obtained because of the scarcity of these cells. Quantitation of these cells could only be reliably obtained at the edges of GFAP$^+$ and mixed explants where single cells of this type could be identified (Table 3).

Table 2—Area/Perimeter (A/P) Ratios of FN$^+$ and GFAP$^+$ Cells From Gliomas at Explantation

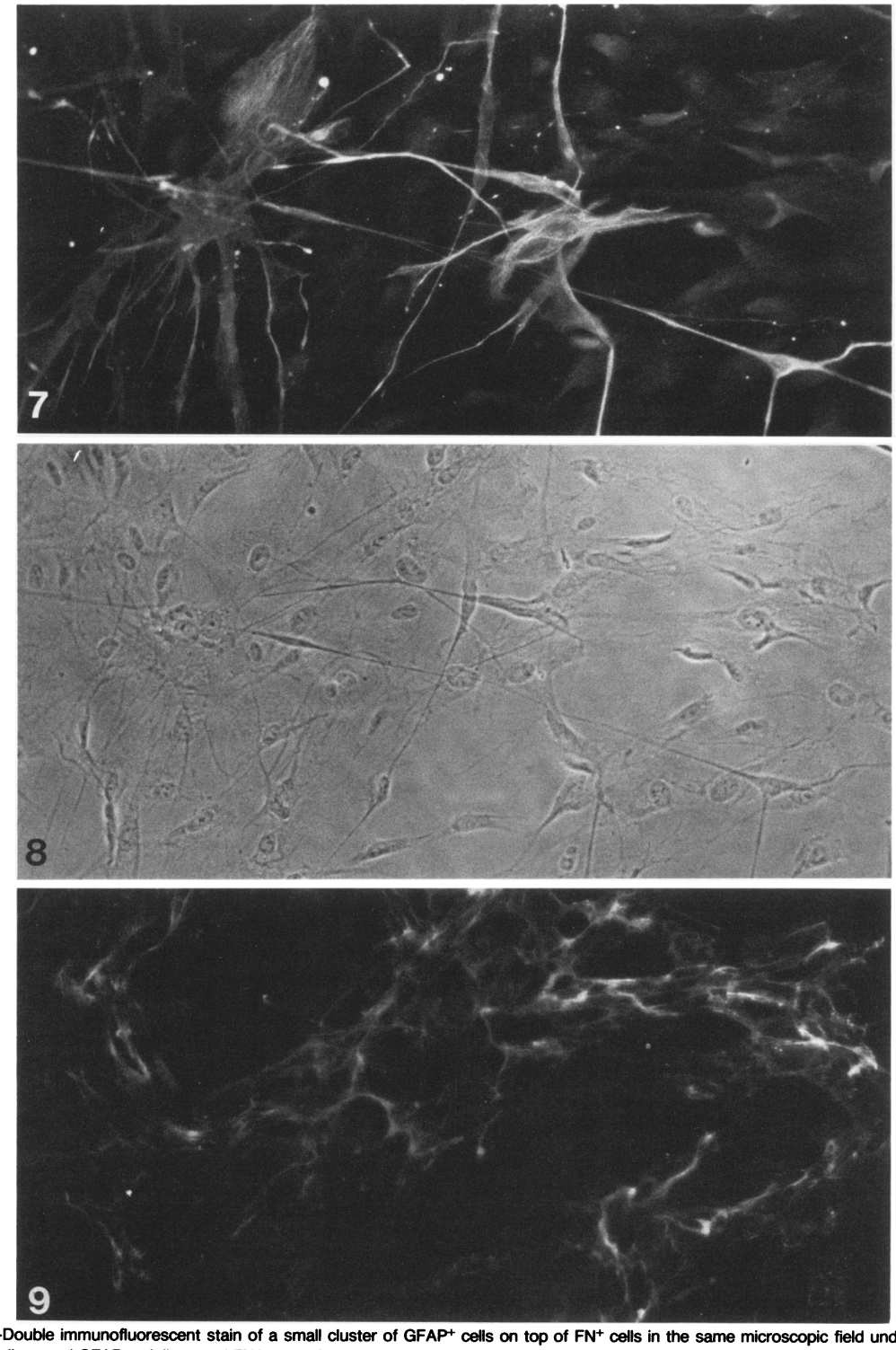
Case	FN$^+$ cells	GFAP$^+$ cells
6a*	15.0 (20)†	9.0 (14)
7	14.9 (19)	11.7 (13)
8	39.4 (20)	10.9 (20)

*Same cases described on other tables share identical case numbers. A different flask grown from Case 6 was used for measurement of outgrowth diameters (Table 1). Different flasks from Cases 7 and 8 were trypsinized and analyzed at later passages (Table 5).

†The numbers represent the medians for each group. The number of individual cells analyzed is in parentheses. A/P ratios of FN$^+$ cells were compared with ratios of GFAP$^+$ cells in each case by the Wilcoxon rank sum test. All three cases were significant at the $P < 0.005$ level.

Quantitation of Growth Around Explants

In order to determine whether FN$^+$ and GFAP$^+$ cells manifested different growth at explantation, we harvested explanted gliomas at time intervals in culture, and explants seeding FN$^+$ cells were measured apart from explants seeding GFAP$^+$ cells. Four of the six gliomas seeded GFAP$^+$ cells and FN$^+$ cells around separate explants of the same glioma (Table 1).



Figures 7–9—Double immunofluorescent stain of a small cluster of GFAP$^+$ cells on top of FN$^+$ cells in the same microscopic field under three different illuminations. Indirect anti-GFAP and direct anti-FN immunofluorescence. (×300) **Figure 7**—Illuminated with 534–558-nm excitation of indirect rhodamine anti-GFAP. **Figure 8**—Illuminated by phase contrast. **Figure 9**—Illuminated with 440–490-nm excitation of direct fluorescein anti-FN.

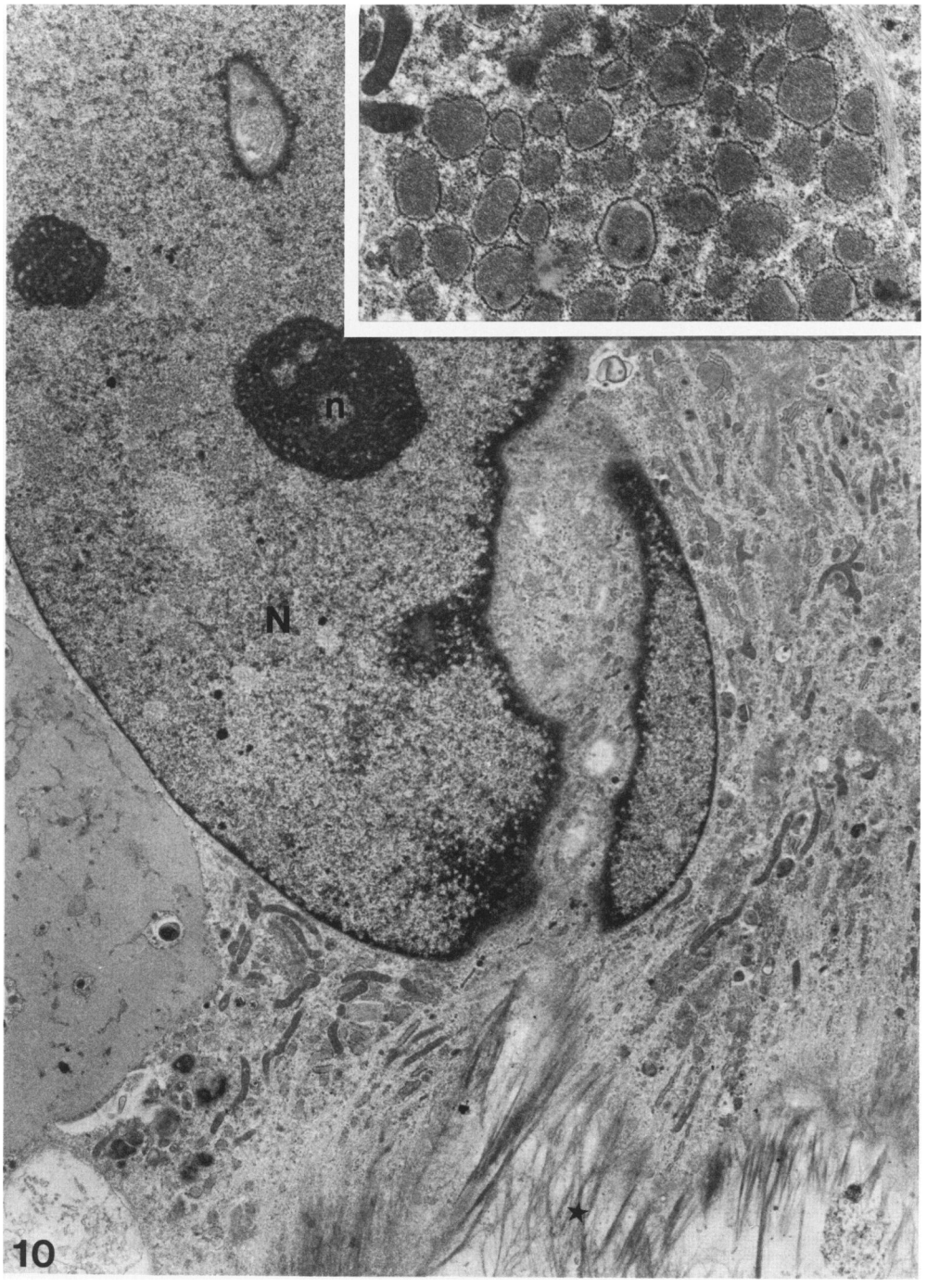


Figure 10—Extracellular filaments of intermediate electron density (*star*) coat the surface of an FN^+ cell, which has a convoluted nucleus (*N*) and several cross-sections of dense nucleolar material (*n*). Rough endoplasmic reticulum is filled with granular material (**Inset**). (Transmission electron micrograph of specimen stained with uranyl acetate and lead citrate, ×4200; **Inset**, ×15,000)

Among these explants, the mean diameter of the ring of the FN^+ cells around all 12 FN^+ explants was 9.2 mm ± 5.2 SD. From the same four gliomas, the mean diameter of the ring of $GFAP^+$ cells around all eight $GFAP^+$ explants was 2.0 mm ± 1.3 SD, significantly smaller ($P < 0.005$) than comparable measures of growth of FN^+ cells (Table 1).

The rate of growth of FN^+ and $GFAP^+$ cells around explants was estimated from individual and mean values of all diameters of each type of explant at each

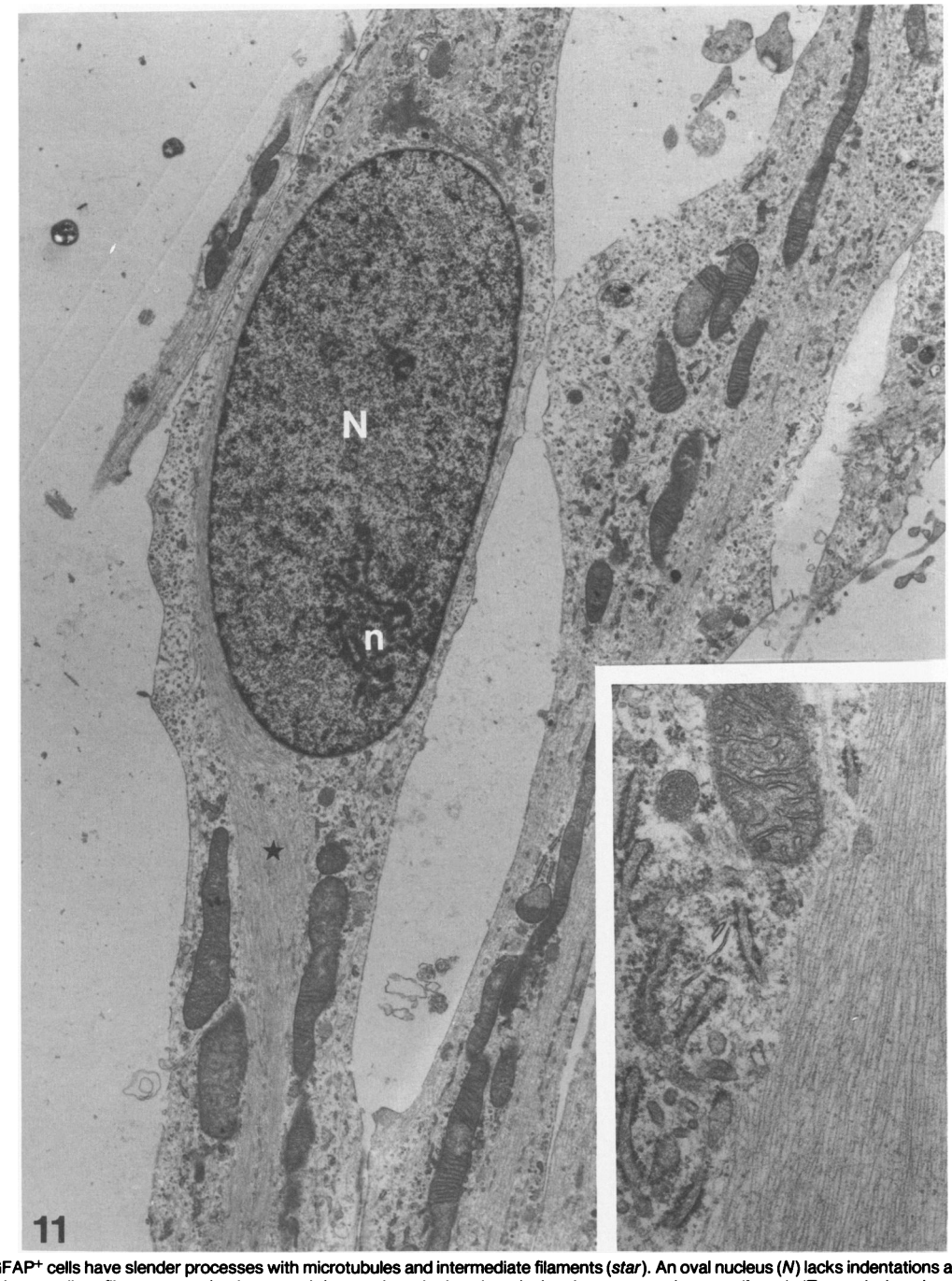


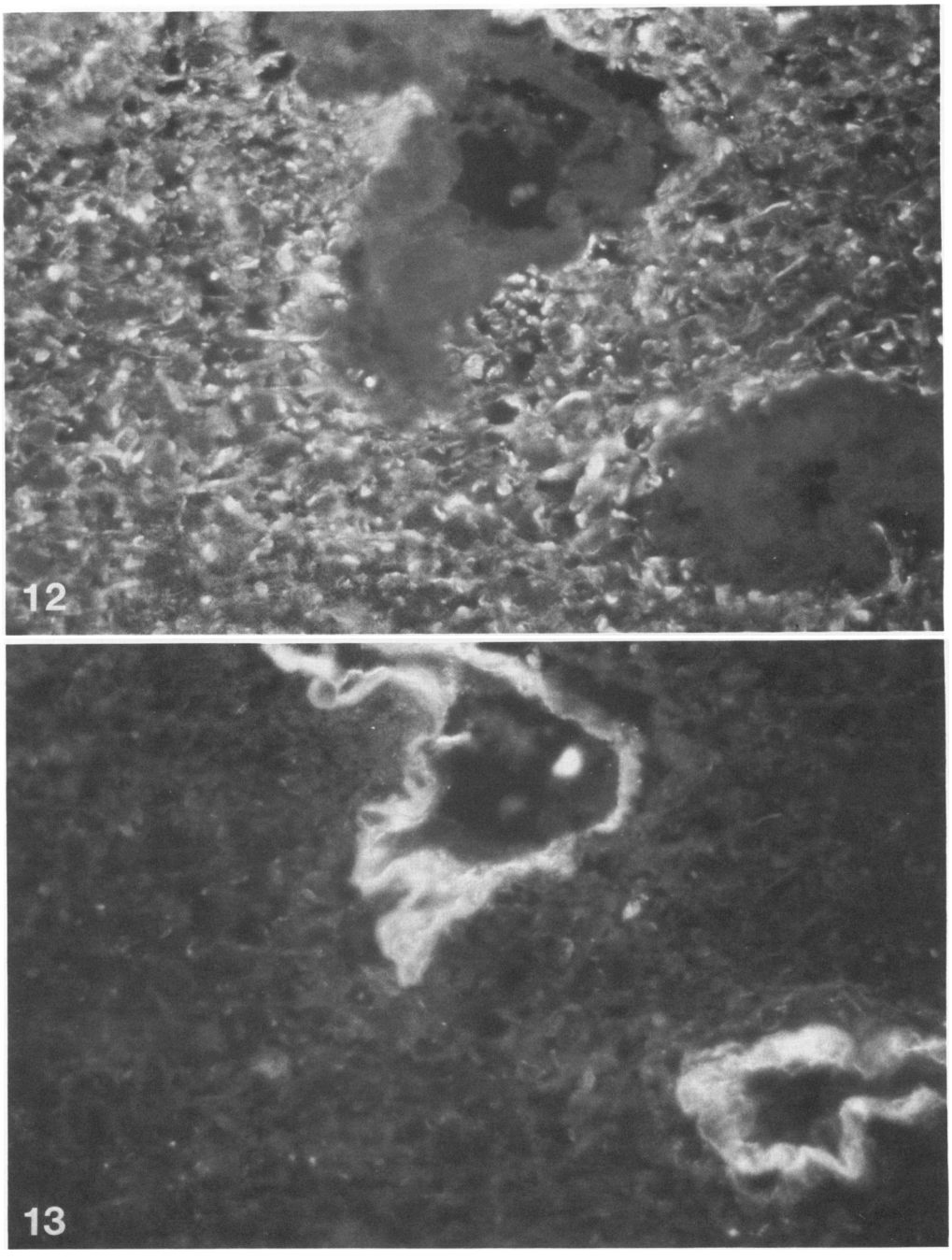
Figure 11—GFAP$^+$ cells have slender processes with microtubules and intermediate filaments (*star*). An oval nucleus (*N*) lacks indentations and has a spread nucleolus (*n*). Intermediate filaments predominate, and the rough endoplasmic reticulum has narrow cisternae (**inset**). (Transmission electron microscopy, uranyl acetate and lead citrate, ×6300; **inset**, ×30,000)

time interval sampled. The increase was higher in FN$^+$ cells than in GFAP$^+$ cells (Figure 16 and Table 4).

Cells in Culture Passages Subsequent to Explantation

Six of seven gliomas in their second passage had no GFAP$^+$ cells detectable by immunofluorescence (Table 5). This was in marked contrast to explants where five of six gliomas had growth of GFAP$^+$ cells around explants (Table 1). Loss of GFAP$^+$ cells (or staining characteristics) during culture of most gliomas did not occur at explantation, but rather at very early culture passage after explantation.

One glioma clearly had many GFAP$^+$ cells in its second passage, and another had rare GFAP$^+$ cells at

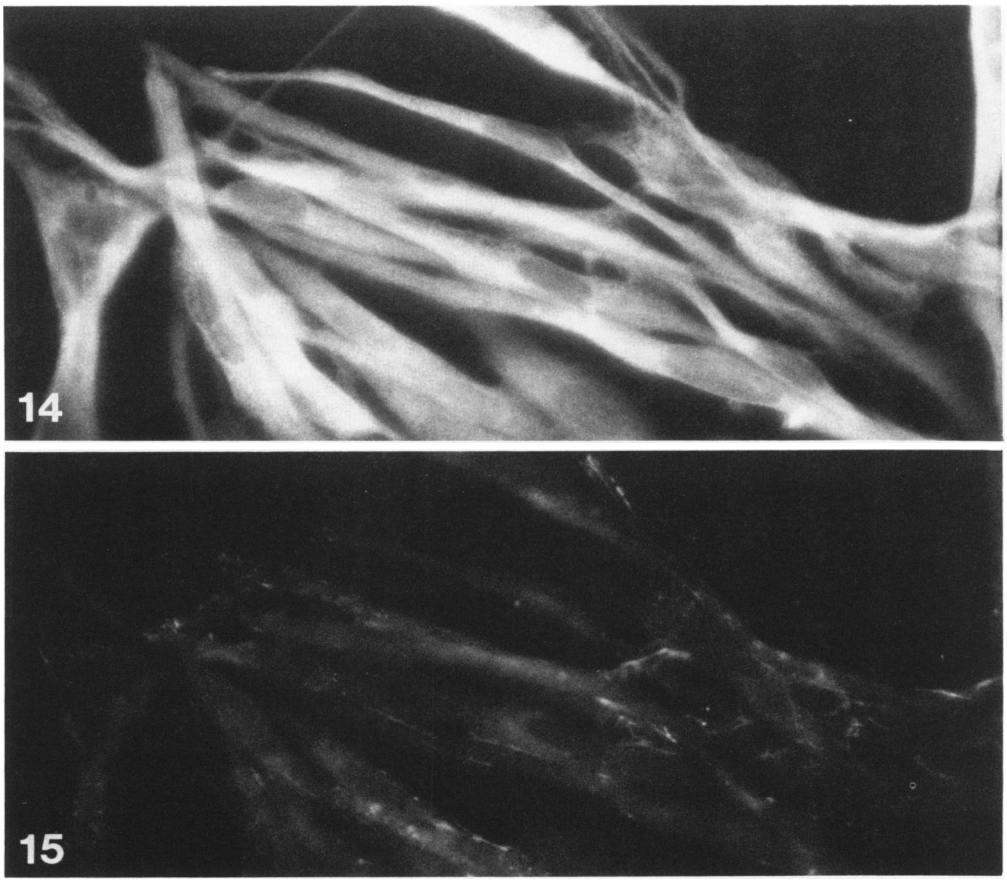


Figures 12–13—Tissue of the glioma which provided the explants shown in Figures 1–3. Same field stained by double immunofluorescence for FN and GFAP under different illuminations. (×200) **Figure12**—Excitation of indirect rhodamine anti-GFAP shows that the parenchyma contains $GFAP^+$ cells. Only a few of the $GFAP^+$ cellular processes have been sectioned longitudinally, whereas the others are in cross and tangential sections. **Figure 13**—Excitation of direct fluorescein anti-FN shows that endothelial proliferations contain FN^+ cells.

passage 20. Neither event was common in this series.

Of the 23 total cases including explants examined in this series, only 2 cases showed predominant growth of $GFAP^+$ cells, as compared with FN^+ cells at the time they were sampled. These were Cases 2 and 14.

In contrast to $GFAP^+$ cells, FN^+ cells were abundant from most gliomas after first passage. Their fluorescence-, light- and electron-microscopic features were the same as those of FN^+ cells at explantation, and they were the same from different types of high grade gliomas and from gliosarcoma.



Figures 14 and 15—Double-immunofluorescence stain for FN and GFAP of a rare cluster of cells staining for both antigens. (×480) **Figure 14**—Excitation of indirect rhodamine anti-GFAP. **Figure 15**—Excitation of direct fluorescein anti-FN.

Discussion

The following observations require special emphasis either because they have not been generally appreciated or because they provide clues to the mechanism of altered phenotypic expression in gliomas.

1. Phenotypic alterations occur earlier and more completely than previously noted. One study has shown disappearance of GFAP$^+$ cells during the first 12 passages *in vitro*.[6] The present study shows loss of GFAP$^+$ cells in 6 of 7 cases examined in the second passage. While GFAP$^+$ cells grew from most gliomas at explantation, they predominated over FN$^+$ cells in only 2 of all 23 cases studied. FN$^+$ cells occur at least as soon as 1½ weeks after explantation. These factors should be appreciated in the design of research and clinical studies utilizing primary cultures of patients' gliomas. The ubiquity of FN$^+$ cells in both primary and established glioma cell lines underscores the need to understand these cells and their relationship to gliomas *in situ*.

Table 3—Expression of FN and GFAP by Single Unconnected Cells Near GFAP$^+$ and Mixed Explants*

Cell type	Number of cells	Percentage of cells counted
FN$^+$	32	36
GFAP$^+$	38	43
FN$^+$/GFAP$^+$	7	8
Negative	12	13

*All counts were done on Case 6 (Table 1), which was the only case with double FN$^+$/GFAP$^+$ cells at explantation. These regions were selected for counting by virute of the certainty with which single cells could be classified and enumerated. Single positive cells around FN$^+$ explants were predominantly FN$^+$.

2. Most FN$^+$ cells are clearly different from GFAP$^+$ cells by multiple criteria. The results indicate that, in all but the single case noted below, two distinct populations of cells defined by two different constellations of shape, ultrastructural features, and content of GFAP or FN grow out from explants of individual gliomas. The present findings strongly reinforce the theory that the two different cell populations in glioma explants display fairly consistent antigenic

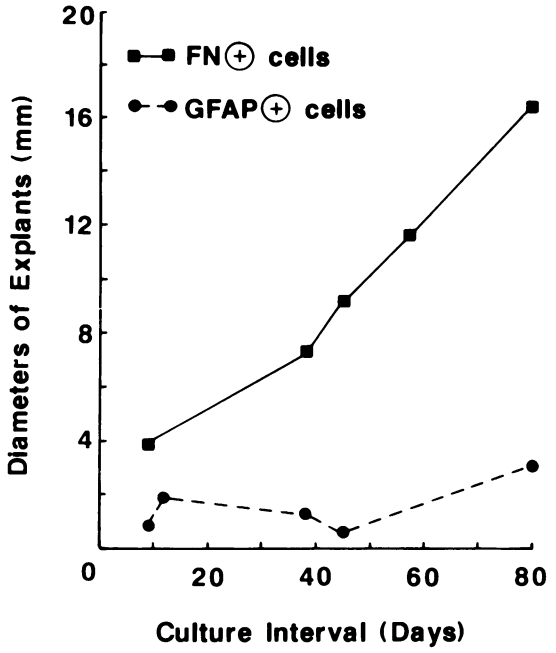


Figure 16—Diameters of cells around explants after various intervals of culture.

and morphologic differences and therefore consist of 1) actively proliferating mesenchymal cells which are FN^+, and 2) $GFAP^+$ glial cells, which have a tendency to grow on the FN^+ cells, using the latter as a "feeder layer." This "feeder layer" interaction may reflect interactions *in situ*.

3. In contrast to complete separation and propagation, which is rare,[8,23,24] FN^+ cells are partially separable from $GFAP^+$ cells on a regular basis during explantation.

4. FN^+ cells grow faster than $GFAP^+$ cells. Since the FN^+ cells in passage 2 of glioma culture probably come from FN^+ cells at explantation, the most plausible explanation of the usual predominance of FN^+ cells in passage 2 and later passages is their faster growth. Diameters of the rings of FN^+ cells, compared with $GFAP^+$ cells, around explants support this possibility.

5. The tendency toward mutually exclusive expression of FN and GFAP by gliomas is strong but not absolute. Mutually exclusive expression of FN and GFAP, previously noted among human glial and glioma cell lines and primary cultures of fetal and neonatal brain,[16-22] occurred here in explants of 5 out of 6 human gliomas, and in all 17 primary glioma cultures. However, the single case with individual

Table 4—Comparison of Mean Diameters of Cellular Outgrowths From Explants

Case number	Days in culture	Predominant cell type*	Mean diameter of ring of cells (mm) ± SD
2	12	$GFAP^+$	2.0 ± 0.6
3	38	FN^+	7.3 ± 3.9
4	45	FN^+	9.3 ± 5.2
		$GFAP^+$	0.6 ± 0.1
5	57	FN^+	11.7 ± 7.4
6	80	FN^+	16.5 ± 2.1
		$GFAP^+$	3.2 ± 0.7

*Only explants with cells which were predominantly (less than 2% contamination with the other cell type) either FN^+ or $GFAP^+$ were quantitated.

Table 5—Expression of FN and GFAP by Glioma Cells in Primary Culture Passages After Explantation

Case number	Diagnosis	Passage number	FN^+ cells	$GFAP^+$ cells
9	Astrocytoma, Grade III	1	Abundant*	< 1%
9		2	100%	None detected
10	Mixed oligondendroglioma-astrocytoma, Grade II-III	2	100%	None detected
11	Glioblastoma multiforme	2	100%	None detected
12	Glioblastoma multiforme	2	Abundant	None detected
13	Astrocytoma, Grade II-III	2	Abundant	None detected
14	Astrocytoma, Grade IV	2	5%	35%
15	Astrocytoma, high grade	2	100%	None detected
7	Astrocytoma, high grade	3	100%	None detected
8	Glioblastoma multiforme	3	Abundant	None detected
16	Astrocytoma, Grade III	3	95%	None detected
17	Astrocytoma, high grade	4	100%	None detected
18	Astrocytoma, Grade IV	4	80%	(9%)†
19	Astrocytoma, high grade	8	50%	None detected
20	Gliosarcoma	8	Abundant	None detected
8	Glioblastoma multiforme	10	Abundant	None detected
21	Glioblastoma multiforme	20	Abundant	< 1%
22	Glioblastoma multiforme	22	Abundant	None detected
23	Astrocytoma, Grade III	23	Abundant	None detected

*This terminology was used when positive cells were plentiful but packed closely among negative cells, which made them uncountable.
†Weak staining barely above background was interpreted as positive by one of two observers.

cells carrying both FN and GFAP markers implies that mutually exclusive expression is not absolute in these early cultures. This result might be explained by redistribution of FN from FN$^+$ cells to GFAP$^+$ cells. However, the redistribution explanation must also explain why no dual-labeled cells occurred among the other 7 gliomas which grew both FN$^+$ and GFAP$^+$ cells. These dual-labeled cells raise questions about a stem cell seeding both FN$^+$ and GFAP$^+$ cells. Although it is difficult to rule out the contribution of a stem cell with potential for dual expression to the mechanism of changing glioma antigens either at an earlier stage of tumor progression *in situ* or as a second mechanism, the preponderance of evidence in this study is that overgrowth of a preexisting mesenchymal cell population is sufficient to explain the occurrences in the majority of cases during the time intervals studied here.

Previous studies of the explantation phase of glioma culture have emphasized the possibility of fibroblast growth.[25,26] At least some of these cells are not normal fibroblasts, but are actively proliferating, supportive cells. Their proliferative capacity *in vitro* has been established to be appreciably greater than that of the primary glioma cells.[26] This issue is further addressed in other studies[8,10,13,14,25,27,28] and reviewed here.

Although fibroblasts are FN$^+$ and may have a role in the culture of gliomas, certain repeatedly observed phenomena cannot be explained solely by the growth of fibroblasts: 1) Cells cultured from gliomas including numerous FN$^+$ cells which lack GFAP are not always diploid.[8,10,14,29-31] Fibroblasts are diploid. 2) The marker proteins and ultrastructure of most established glioma cell lines resemble the FN$^+$ cells more closely than the GFAP$^+$ cells from explants in this study. Similarities between established gliomas and FN$^+$ cells include rough endoplasmic reticulum with dilated cisternae, 6–7-nm filaments on the cytoplasmic side of the plasmalemma, loose arrays of intermediate filaments, extracellular filaments, and relatively flat, polygonal shape.[32-37] Established glioma lines are neoplastic by multiple criteria.[10,32-38] 3) There is evidence from both cytologic study and culture in low percentages of fetal calf serum for neoplasia of FN$^+$ cells around explants of gliomas.[27,28]

Similarities between GFAP$^+$ glioma cells and glial cells are clear from various features defined here, not the least of which is the characteristic presence of GFAP. In contrast, similarities of FN$^+$ cells to other cells of known histogenetic origin are only beginning to be understood. Primary and established FN$^+$ glioma cells express mesenchymal and epithelial, but not endothelial, markers.[12,14] In this respect, their phenotypes are similar to the predominant cell type in normal human brain cultures, which may be leptomeningeal cells.[39] Although previously observed desmosomes in glioma cultures[12] are yet another feature shared with leptomeningeal cells, the rarity of desmosomes found in this study of cells from gliomas suggests incomplete expression of leptomeningeal features or the contribution of at most a minor leptomeningeal subpopulation. Similarities between the proliferating vascular elements of gliomas *in situ* and FN$^+$ cells in culture strongly suggest that FN$^+$ cells may be derived from the proliferating vascular mesenchymal elements of gliomas. As compelling as this circumstantial evidence of marker similarity may be, no experiment has absolutely proven the origin of FN$^+$ cells from these elements.

Two distinct populations of cells at explantation provide an opportunity to study, under *in vitro* conditions, the biology of two cell types derived from one glioma, which may be clinically important for different reasons: 1) GFAP$^+$ cells are constituents of the parenchyma of most gliomas *in situ*.[4-7,40-45] GFAP$^+$ neoplastic glia infiltrate the central nervous margins of gliomas and may be the principal reason for the failure of surgery to cure gliomas.[46,47] Their importance to immunotherapy is suggested by the longer survival of patients inoculated with GFAP$^+$ cells.[48] 2) FN$^+$ cells increase in higher grade and less differentiated gliomas. Their effect on patient survival is rarely as evident as when they become sarcomatous *in situ*.[2,3] One gliosarcoma showed a FN$^+$ cell population within the neoplasm at biopsy which over the next year overgrew the GFAP$^+$ cells and caused the patient's demise.[3] Efforts to identify similar subpopulations of malignant FN$^+$ cells in other types of high-grade gliomas are under way.[49]

Two distinct cellular subpopulations could be part of the substantial heterogeneity observed between glioma cell lines from different patients.[8,10] For example, it might explain the occurrence of the unusual cell line U-251 MG. This glioma line is GFAP$^+$, and its production of FN is either low[8,10] or undetectable.[16,50]

Vimentin is an intermediate filament component more prominent in mesenchymal than in many other types of cells. Vimentin is low to inconspicuous in reactive GFAP$^+$ astrocytes not at the edge of a lesion.[51,52] Vimentin coexists with GFAP in some gliomas[51-58] and exists without GFAP in sarcomatous portions of gliosarcomas.[54] It has been suggested that increases in the ratio of vimentin to GFAP correlate with degrees of malignancy in astrocytomas.[58] Studies of vimentin staining *in situ* and *in vitro* could provide valuable insights into questions of antigenic changes in gliomas over a time period and their possible rela-

tionship to degrees of malignancy as well as to subpopulations of glioma cells.

Understanding the mechanism of antigenic instabilities in glioma cells is important to understanding the biology of these devastating tumors *in situ*. If neoplastic glia can alter their phenotypes in a multiplicity of different ways responding to different stresses, the potential of glioma cells to repeatedly escape therapeutic challenges is likely. The present study suggests an alternative possibility that apparent antigenic instability simply reflects overgrowth of one cell type from a small assortment of preexistent cell types. Should this be the case, therapeutic approaches may need only to aim toward identifying and destroying the offensive cell types in this assortment to eradicate the glioma.

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