Electron Microscopic Observations on Human Glioblastomas and Astrocytomas Maintained in Organ Culture Systems

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The fine structure of four glioblastomas and two cerebellar astrocytomas maintained in organ culture systems up to 137 days and 43 days, respectively, using either a three-dimensional sponge foam matrix technic or a Millipore filter platform technic, is described and compared. The cells of both tumor types showed increased astrocytic differentiation, characterized by a progressive increase in glial filaments associated with an increase in free ribosomes and granular endoplasmic reticulum. A progressive increase in basement membrane material, presumably originating from explanted endothelial cells or pericytes, was also found in both tumor types and was often associated with increased numbers of collagen fibrils. Astrocytic tumor cell processes frequently preserved their contact with this basement membrane material. Microvascular fenestrations or gaps in endothelial cells were not identified. These electron microscopic features appear to correspond to the early stages of perivascular sclerosis previously noted by light microscopy in gliomas maintained in organ culture systems and are presumably related to the progressive obliteration of the functional microvasculature (Am J Pathol 73:589–606, 1973).

THE FEASIBILITY OF ORGANOTYPIC CULTURE METHODS for the growth and maintenance of human glioblastomas *in vitro* has been reported, with a description of the light microscopic evolution of five examples using either a three-dimensional sponge foam matrix technic or a Millipore filter platform technic.[1] The chief advantage of these organ culture systems is that they permit the retention of the tissue organization of the original neoplasms. The cellular features in these cultures faithfully reproduce those of the tumors *in vivo,* while the migration and proliferation of fibroblasts in the outgrowth zone of the explants are suppressed.

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This report will describe the electron microscopic features of a series of human astrocytic gliomas maintained in organ culture systems, including those of sister cultures of three of the glioblastomas previously described by light microscopy. In this series, the *in vitro* evolution of two well-differentiated, histologically benign cerebellar astrocytomas will be compared to that of four anaplastic glioblastomas, with special reference to the progressive increase of glial filaments and to the production of increased perivascular basement membrane material in the explanted neoplasms.

Materials and Methods

Material

Specimens were obtained at craniotomy from four glioblastomas (three temporal and one parietal) from adults ranging from 42 to 79 years of age, and from two cerebellar astrocytomas from males of 3½ and 24 years of age. Part of the tissues was immediately immersed in glutaraldehyde and trimmed into cubes 1 mm on an edge for electron microscopic study as detailed below. Other fragments were immersed in sterile Hank's IX balanced salt solution containing 50 units/ml of penicillin and subsequently processed for culture on gelatin sponge foam matrices and on Millipore filter platforms. Details of the culture technics, the conditions of incubation and the monitoring and tissue processing methods have previously been described.[1] Separate fragments from the same material were set aside for routine formalin fixation and histologic examination.

Maintenance in Organ Culture Systems

Viable cultures from the four glioblastomas were maintained up to 20, 21, 91 and 137 days on sponge foam and up to 10, 13, 20 and 122 days on Millipore filter. Viable cultures from the two cerebellar astrocytomas were maintained for up to 37 and 43 days on sponge foam and up to 18 and 29 days on Millipore filter.

Electron Microscopic Methods

Fragments of the original tumors were fixed in chilled 3.5% glutaraldehyde in 0.07 M phosphate buffer (pH 7.0, 550 mOsm) for periods of 2 to 6 days. The tissue was then washed in 0.045 M phosphate buffer with sucrose (pH 6.9, 340 mOsm) for 1 to 12 hours. Osmication, dehydration through graded ethanols and embedding in Epon 812 were carried out according to established methods.

Specimens from the four glioblastomas were selected for electron microscopic study at weekly intervals up to 122 days and from the two cerebellar astrocytomas up to 43 days *in vitro*. The cultures were fixed in chilled 2% glutaraldehyde in 0.05 M phosphate buffer (pH 6.9, 340 mOsm) for 1 hour, and processed and embedded as above. A few cultures were alternatively fixed in chilled 2.5% glutaraldehyde-2% paraformaldehyde in 0.2 M cacodylate buffer pH 7.2), followed by 5% glutaraldehyde-4% paraformaldehyde in cacodylate buffer (pH 7.2) for 1 hour. After rinsing in cacodylate buffer, the blocks were further fixed in cacodylate-buffered osmium tetroxide, followed by aqueous uranyl acetate.[2] Dehydration was carried out in graded methanols, and the tissue was embedded in Epon 812.

One-micron-thick sections were stained with Mallory's Azure II-methylene blue,[3] and thin sections were collected on coated or uncoated copper grids and stained with uranyl acetate [4] and lead citrate.[5] The tissues were examined in a Siemens IA electron microscope operating at 80 kV with a 30- or 50-μ copper foil objective aperture.

Results

Light Microscopy Observations

The original microscopic appearances and the sequential evolution in organ culture of three of the four glioblastomas in this group (T4, T10 and T18) have previously been described in detail.[1] A fourth example (T44), maintained for 20 days *in vitro* and not described in the previous report, showed essentially similar features. In organ culture systems, all four tumors demonstrated a progressive increase in astrocytic differentiation, which became most marked after 3 to 6 weeks. Explants maintained on sponge foam showed rapid (within 7 days) and extensive invasion of the matrix. Mitotic figures and occasional giant cells were observed. The explants did not exhibit focal necrosis, pseudopalisading or vascular endothelial proliferation that are characteristically found *in vivo*. As previously noted, perivascular sclerosis with gradually increasing acellular hyalinization of the blood vessel walls was a conspicuous feature.

The two cerebellar astrocytomas were originally composed of small astrocytic cells which were arranged in focally compact, fibrillated areas alternating with more loosely textured and poorly fibrillated areas. Mitotic figures, giant cells, necrosis or other features suggestive of anaplasia were absent. In cultures on sponge foam, the astrocytomas showed a slight to moderate degree of invasion of the matrix (Figure 2), with excellent preservation of the general histologic organization seen *in vivo*. The dual pattern of compact fibrillary foci and of loosely arranged reticulated areas was preserved in both sponge foam and Millipore filter cultures (Figure 1).

After 2 weeks *in vitro*, a number of tumor cells showed a marked increase in eosinophilic cytoplasm, with the occasional presence of a delicate ground-glass pattern suggesting small lipid droplets, and an eccentric displacement of their nuclei (Figures 1 and 2). These cells, which resembled the gemistocytic astrocytes seen in cerebral astrocytomas, demonstrated fibrils that extended from the periphery of the cytoplasm and stained positively with Mallory's phosphotungstic acid hematoxylin. After approximately 2 weeks *in vitro*, the thickness and compactness of the neuroglial fibrillary network in the explanted neo-

plasms was increased (Figure 2) when compared with the original tumor specimens or with the earlier stage of culture (Figure 1). As with the glioblastomas, perivascular sclerosis of the supporting blood vessels was observed.

Electron Microscopic Observations

Original Tumor Specimens

The astrocytic nature of the tumor cells from the glioblastomas was sometimes evident from the presence of small wispy bundles of cytoplasmic filaments measuring 80 to 90 Å in diameter dispersed among other cytoplasmic organelles (Figure 3); however, many of the tumor cells had very few filaments. The cells generally had large pleomorphic nuclei with prominent nucleoli and exhibited multiple nuclear infoldings and cytoplasmic invaginations. The cytoplasm contained cisternae of Golgi apparatus, scanty granular endoplasmic reticulum and free ribosomes, mitochondria and membrane-bound dense bodies, presumably lysosomes. The more distal cell processes contained stout bundles of glial filaments.

In general, the cells in the cerebellar astrocytomas resembled nonneoplastic astrocytes with their normal complement of cytoplasmic filaments, most of which measured 80 to 90 Å in diameter. Other organelles, such as Golgi apparatus, granular endoplasmic reticulum, mitochondria, lysosomes and microtubules measuring 200 to 240 Å in diameter, were dispersed throughout the cytoplasm. In contrast to those of the glioblastomas, the cell nuclei were round or oval and showed a conspicuous absence of folding or tortuosity of the nuclear membrane. In both tumor types, the lumens of the vascular channels appeared reduced because of the presence of numerous plump endothelial cells which protruded into them. The endothelial cells were joined by intact tight junctions, and no microvascular fenestrations or gaps were seen.

Organ culture systems

Most of the cells composing the glioblastomas in culture appeared to be medium-sized astrocytes. Typical gemistrocytic astrocytes—*ie*, characterized by abundant cytoplasm filled with tightly compacted mitochondria, ribosomes and 80- to 90-Å glial filaments, were also seen. Differences were, however, observed in the nuclear and some of the cytoplasmic characteristics between the cultured glioblastomas and cerebellar astrocytomas. These essentially recapitulated those already seen in the original tumors. The cells of the glioblastomas regularly

demonstrated folded, reduplicated nuclear membranes which assumed an intricate pattern in the most extreme examples (Figure 4). Cytoplasmic invaginations were frequent and occasionally presented as intranuclear inclusions (Figure 4, inset). Cellular organelles in most instances were unremarkable, except for the appearance of numerous membrane-bound dense bodies (lysosomes), autophagic vacuoles and, in one tumor, numerous giant mitochondria, measuring up to 1.8 μ in diameter, some of which showed a proliferation and disarray of their cristae (Figure 5). Similar giant mitochondria were also present in the original tumor in this case.

The cells of the cerebellar astrocytomas appeared considerably less pleomorphic. The nuclei were uniformly round or oval, and the nuclear membranes showed none of the tortuosity found in glioblastomas (Figure 7). Cellular organelles were morphologically normal.

The cells of both tumor types consistently showed a substantial and progressive increase in cytoplasmic filaments, most of which measured 80 to 90 Å in diameter and which were often arranged in broad bundles (Figure 8). In extreme examples the glial filaments displaced the other cytoplasmic organelles to the periphery of the cell (Figure 6). An association of these filaments with an increase in free ribosomes and granular endoplasmic reticulum was also observed (Figure 8). At high resolution, the substructure of the filaments in cross-section consisted of four globular subunits measuring 21 Å in diameter connected by crossbars and thus forming a tubular structure whose inner lumen measured 25 Å in diameter (Figure 6, inset). As compared with the original tumor, the more distal cell processes showed, on the other hand, no definite increase in the already dense packing of their filaments. Microtubules measuring 200 to 240 Å in diameter were often found in these processes (Figure 9). In an occasional distal cell process of a cerebellar astrocytoma, small aggregates of densely osmiophilic non-membrane-bound material merging with glial filaments and suggestive of Rosenthal fibers [6,7] were noted in a 15-day-old culture maintained on Millipore filter (Figure 9).

Specialized contacts between cell bodies and between cell bodies and processes, resembling puncta adhaerentia,[8] were found in both tumor types (Figure 9).

The perivascular sclerosis previously noted by light microscopy seemed to correspond, in its early stages, to a marked increase of extracellular basement membrane material (Figures 10–12), which often appeared closely associated with the explanted endothelial cells (Figure 10). This feature was consistently found in both tumor types. Fre-

quently, the increased basement membrane material was closely intermingled with an apparent increase of collagen fibrils. It was often heterogeneous in electron density (Figures 11 and 12). Occasionally there was elaborate folding and reduplication of the basement membrane material which did not follow the contours of plasma cell membranes, although it sometimes enclosed irregular membrane fragments (Figure 10). On the other hand, the astrocytic tumor cell processes often preserved their apposition to the outer well-defined borders of this increased basement membrane material (Figures 11 and 12). Isolated cells, probably of mesenchymal origin and thought to represent explanted pericytes or endothelial cells, were sometimes found to be surrounded by a sea of amorphous basement membrane material (Figure 12). Some of these cells contained numerous pinocytotic vesicles.

Discussion

In this study, the sequential fine structural features of two histologically and biologically very distinct types of human astrocytic glioma—four glioblastomas and two cerebellar astrocytomas—were investigated with the objective of seeking possible differences in their capacity for differentiation *in vitro*.

In the original tumors, the obvious cytologic differences seen by light microscopy were, as previously described by others,[9–12] confirmed by electron microscopy. Large nuclei with an intricate folding of the nuclear membrane, dense heavily aggregated chromatin and scanty cytoplasmic glial filaments were characteristic of glioblastomas. In contrast, the cerebellar astrocytomas displayed uniformly rounded or oval nuclei with more evenly dispersed nuclear chromatin and more numerous cytoplasmic glial filaments.

The tumors in organ culture systems yielded three main morphologic findings. The first was the demonstration of progressive gliofibrillogenesis in the cells of both tumor types. Differentiation to highly fibrillary astrocytes was characterized by a substantial increase in broad bundles of 80- to 90-Å cytoplasmic filaments. It was accompanied by an increase of closely associated granular endoplasmic reticulum and free ribosomes, a feature which is well recognized as morphologic evidence of increased protein synthesis and could presumably, in this case, be related to the production of glial acidic fibrillary protein in astrocytic cells.[13,14] The presence of this protein in several gliboblastomas and one cerebral astrocytoma maintained up to several weeks in organ culture systems has been demonstrated by immunofluorescence.[15]

Other *in vitro* systems employed for the study of astrocytic gliomas have similarly demonstrated increased gliofibrillogenesis,[16] but this has not been a universal observation in gliomas maintained either as primary explants or in monolayer or three-dimensional matrix systems.[17–20] On the other hand, the interpretation of cytoplasmic filaments in *in vitro* systems must be subject to caution. In monolayer cultures of a cloned astrocytoma, two types of cytoplasmic filaments have been stated to occur: [20] poorly demarcated filaments measuring 90 to 100Å and microfilaments measuring 75 Å closely packed in long bundles. The latter were considered to be the so-called stress fibers,[20,21] which have long been regarded as a form of structural adaptation to tissue culture environment.[21,22] However, we do not know whether, or to what extent, the *in vitro* conditions of monolayer culture on glass surfaces are comparable to those of three-dimensional organ culture systems.

We are unable, in the present study, to make a clear distinction between these two types of filaments solely on the basis of size. Most of them measured from 80 to 90 Å in diameter, but they ranged from 65 to 100 Å. At high magnification, their end-on view on cross-section showed a substructure consisting of globular subunits connected by crossbars, an appearance identical with that described and illustrated by Wuerker [23] and by Vaughn and Pease [24] as characteristic of glial filaments. These filaments often formed bundles, which are considered to represent, in both protoplasmic and fibrillary astrocytes, the fine structural equivalent of classic neuroglial fibrils.[8] We believe that the cytoplasmic filaments demonstrated in our systems are specific to astrocytic glial cells because of a) the strong affinity of the glial cell processes and intracellular fibrils for the PTAH stain as seen with the light microscope, b) their substructural configuration as described above, c) the positive results obtained by immunofluorescence against the antigen of glial acidic fibrillary protein and d) the occasional association of these filaments with characteristic osmiophilic bodies known as Rosenthal fibers. These bodies have previously been noted in astrocytic gliomas both *in vivo* [6,25] and *in vitro*.[7]

The development of fibrillary differentiation in human astrocytomas and glioblastomas maintained in organ culture systems is therefore comparable to the cellular differentiation known to occur in the normal developing central nervous system as observed in mammalian and avian central nervous system cells maintained *in vitro*. Glial filament formation as a morphologic expression of astrocytic differentiation,[26,27] as well as excessive glial filament production,[28,29] have been demonstrated in these nonneoplastic *in vitro* systems.

The observation that this apparent morphologic expression of differentiation was similar in two biologically very different types of astrocytic glioma seems important. The tendency of organ culture systems to favor differentiation, irrespective of the anaplastic character of the original tumor, stands therefore in contrast to the well-known phenomenon of dedifferentiation which has been abundantly demonstrated in astrocytic gliomas *in vivo*.[25,30]

The second feature of interest in the present study was the increasing production of basement membrane material in the explanted neoplasms. In previous light microscopic observations, the development of progressive perivascular sclerosis in glioblastomas maintained in organ culture systems was described.[1] With electron microscopy, this feature was characterized by an excessive production of amorphous basement membrane material in the perivascular, especially pericapillary, regions. This was often related to increased collagen formation. Distinctive wavy and convoluted patterns of reduplicated basement membrane material similar to those seen in our cultures have been observed in the extracellular space of other tumors of the central nervous system *in vivo* [31] and in acoustic Schwannomas maintained *in vitro*.[32] In our cultures, the increased basement membrane material surrounding the microvasculature was often apposed to the plasma membranes of adjacent astrocytic tumor cell processes, a feature in which the normal gliovascular basement membrane relationship [33] appears accentuated and which has also been noted in the cerebral cortex following experimental local injury.[34]

It is well-known that, in normal circumstances, glial cells share a basement membrane which is related to adjacent mesenchymal tissue such as vascular endothelium.[8,33,35] In this study, the electron-dense borders of the expanded basement membrane material did not invariably invest astrocytic tumor cell processes and were therefore sometimes related solely to the endothelial cells, pericytes or their respective cell processes. Furthermore, only those tumor cell processes adjacent to blood vessels were associated with a basement membrane. It is therefore likely that the production of increased basement membrane material in our organ culture systems was mediated through endothelial cells or closely related cells of mesenchymal origin. This hypothesis appears supported by other circumstantial evidence: in the glia limitans of the developing chick, the production of basement membrane material in association with glial cells appears to be dependent on the presence of mesenchyme.[27] In the myxopapillary ependymoma of the cauda equina, where the ependymal tumor cells are directly apposed to a mesenchymal

stroma derived from the leptomeninges, the glial tumor cells are frequently bordered along their free edges by a basement membrane adjacent to collagen fibrils occupying the extracellular space.[36]

The third feature of note was the failure to demonstrate vascular discontinuities in these human gliomas grown in organ cultures. Microvascular fenestrations and gaps have been reported in human central [37] and peripheral [38] nervous system neoplasms, and in experimental animal gliomas and intracranial sarcomas.[39,40] The absence of these vascular abnormalities in our cultures is presumably related to the lack or, more likely, to the progressive obliteration of a functional microvasculature in the tumor explants maintained in these systems.

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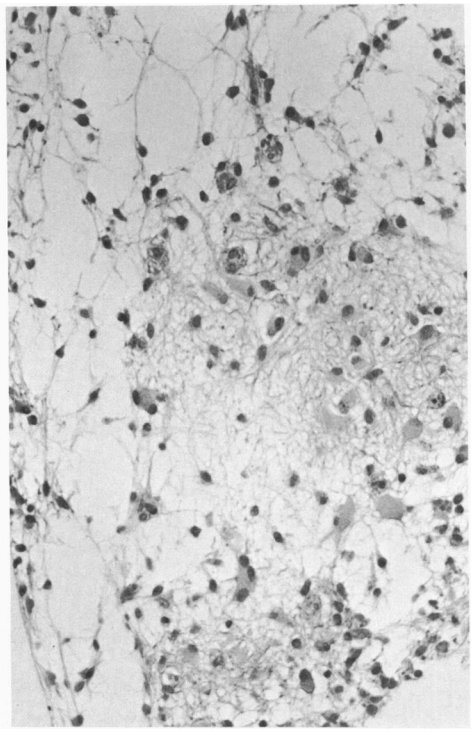
Fig 1—Cerebellar astrocytoma cultured on Millipore filter, 13 days *in vitro*. A dual pattern of fibrillated foci and more loosely arranged areas is seen. Note hypertrophic gemistocytic astrocytes (H & E, × 200).

Fig 2—Culture from same case as Figure 1, on sponge foam, 37 days *in vitro*. Note invasion of sponge foam and increased glial fibrils (H & E, × 300).

Fig 3—Glioblastoma, original tumor. Tumor cell with numerous wispy bundles of 80- to 90-Å cytoplasmic filaments, a tortuous nuclear membrane and peripherally clumped nuclear chromatin. Suboptimal fixation accounts for swollen mitochondria and dilated endoplasmic reticulum (surgical biopsy) (× 9000).



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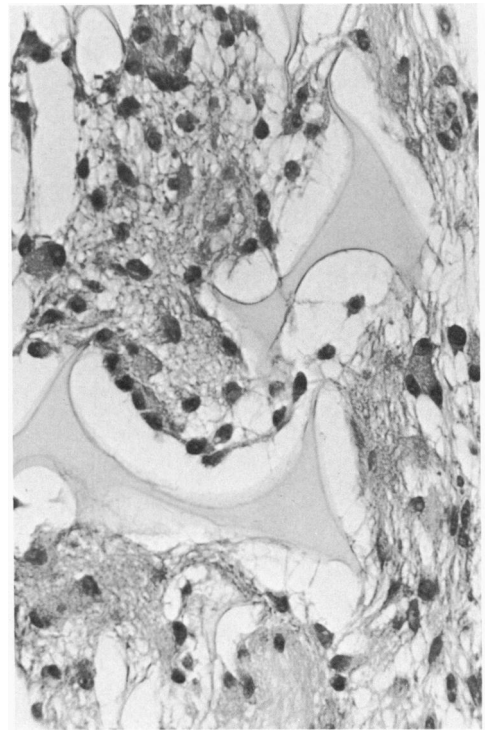

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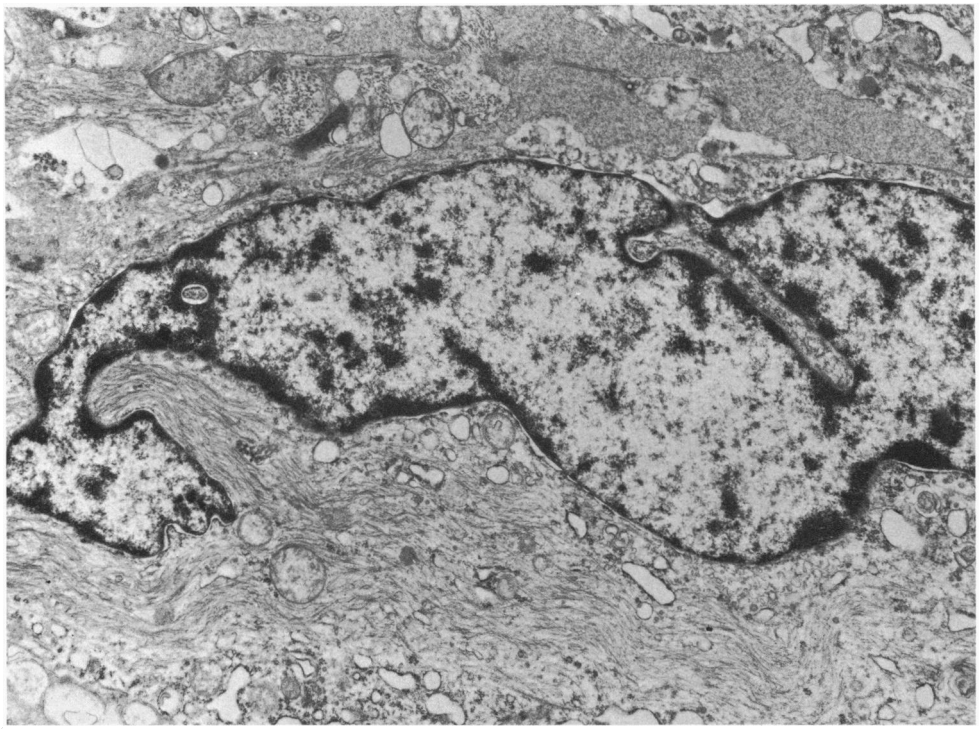

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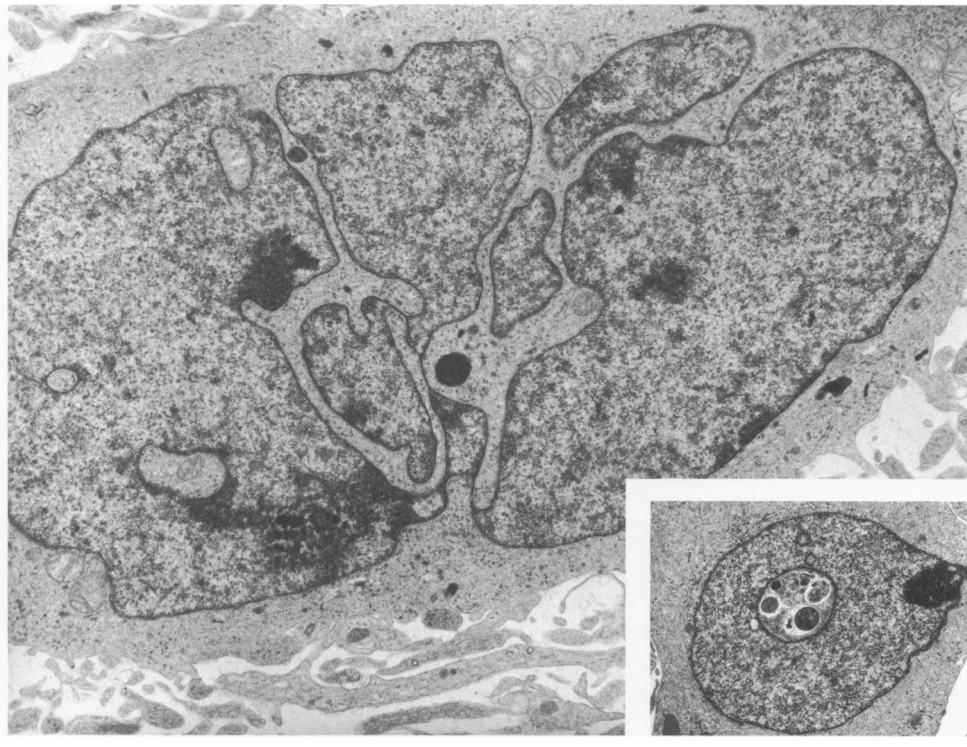

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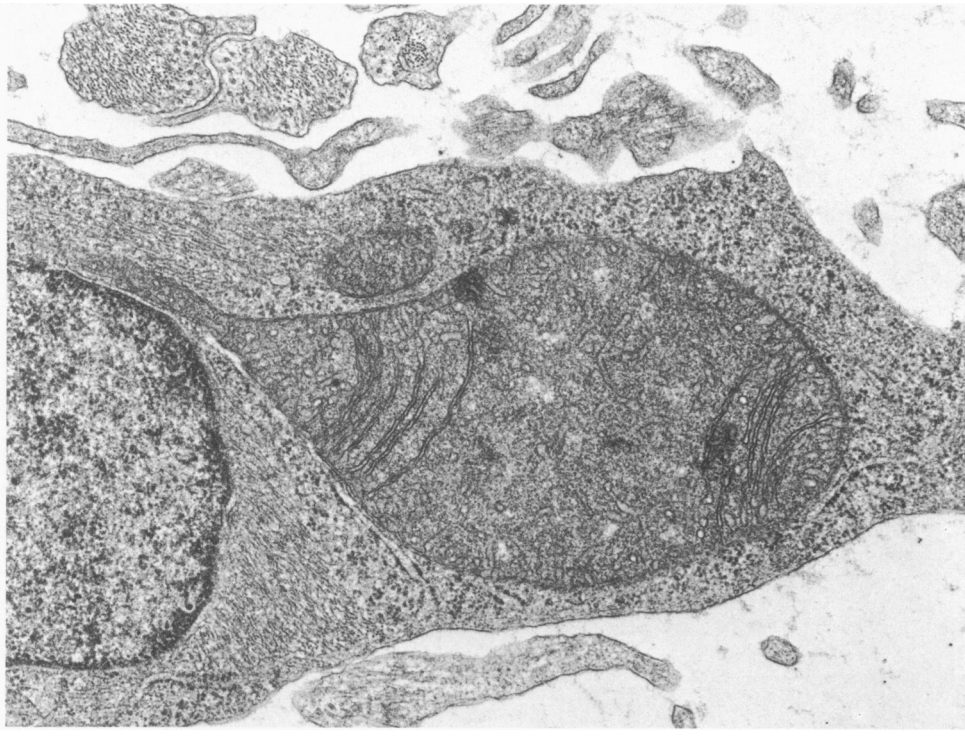

Fig 4—Glioblastoma on sponge foam, 20 days *in vitro.* Tumor cell with a markedly convoluted nuclear membrane (× 9200). **Inset**—A tumor cell showing a central cytoplasmic invagination into the nucleus (× 4250). **Fig 5**—Glioblastoma on sponge foam, 20 days *in vitro.* Tumor cell containing a giant mitochondrion, with irregularly arranged cristae, adjacent to part of the cell nucleus. Note prominent cytoplasmic filaments in this cell and in adjacent cell processes (× 26,000).

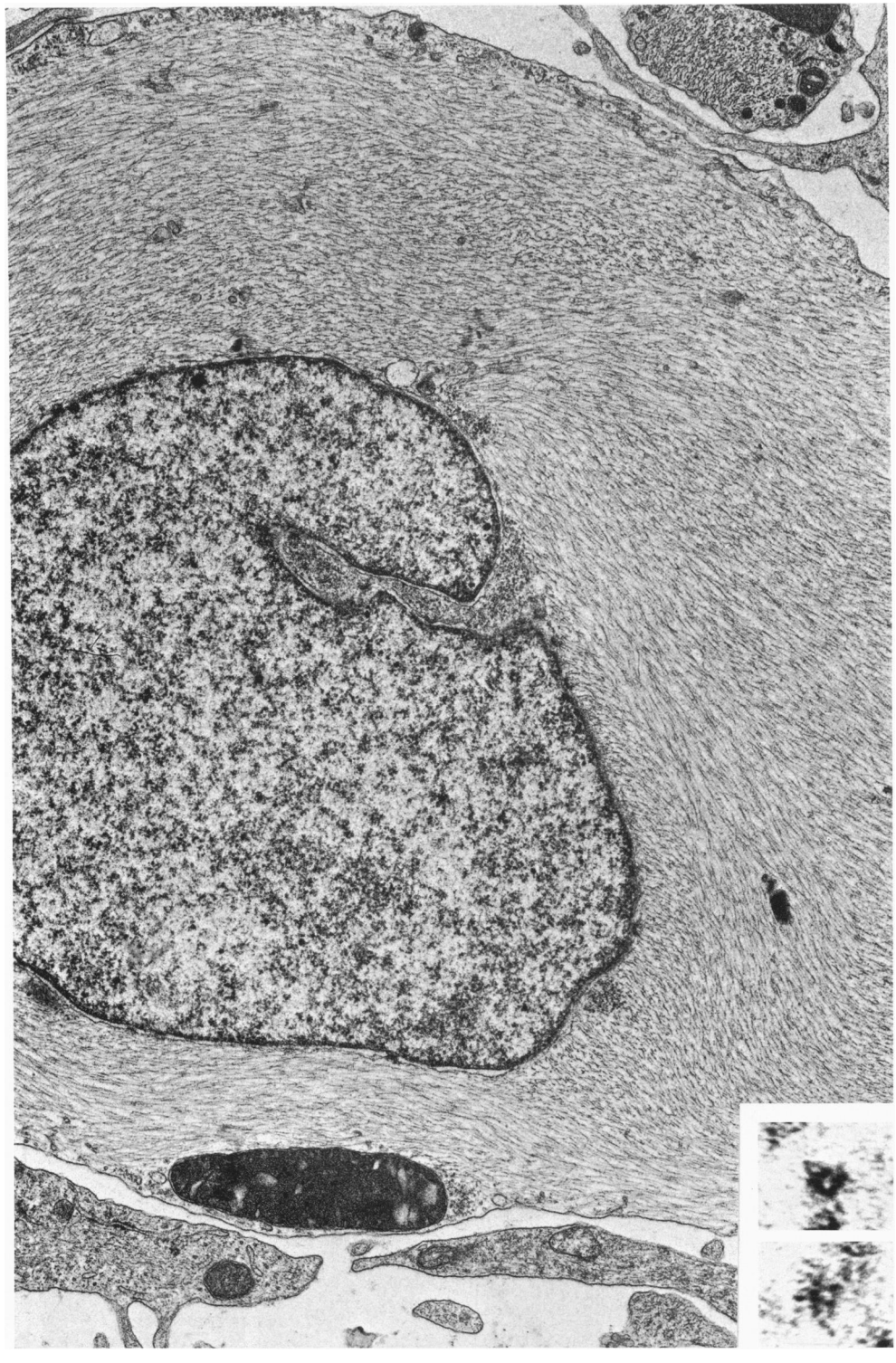

Fig 6—Glioblastoma on sponge foam, 20 days *in vitro*. Tumor cell demonstrating extreme fibrillogenesis with displacement of the other organelles to the periphery (× 27,000). **Inset**—Two glial filaments in cross-section, from another cell process, showing the substructure of the filament wall. The wall is composed of four granules connected by cross-bars, creating an almost circular outline (× 720,000).

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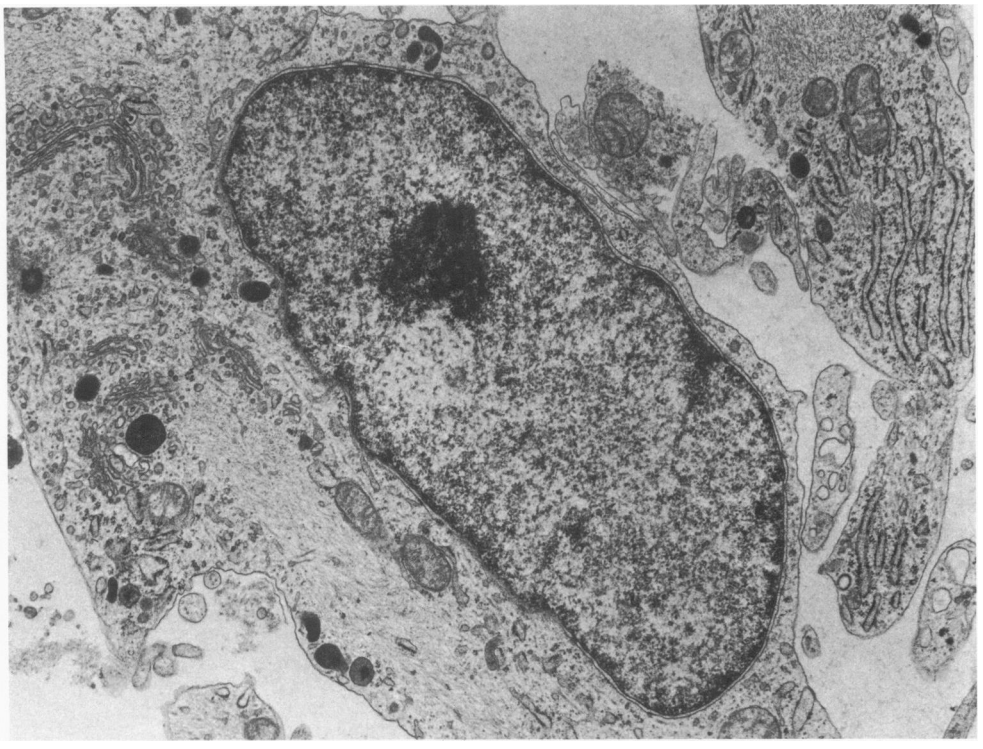

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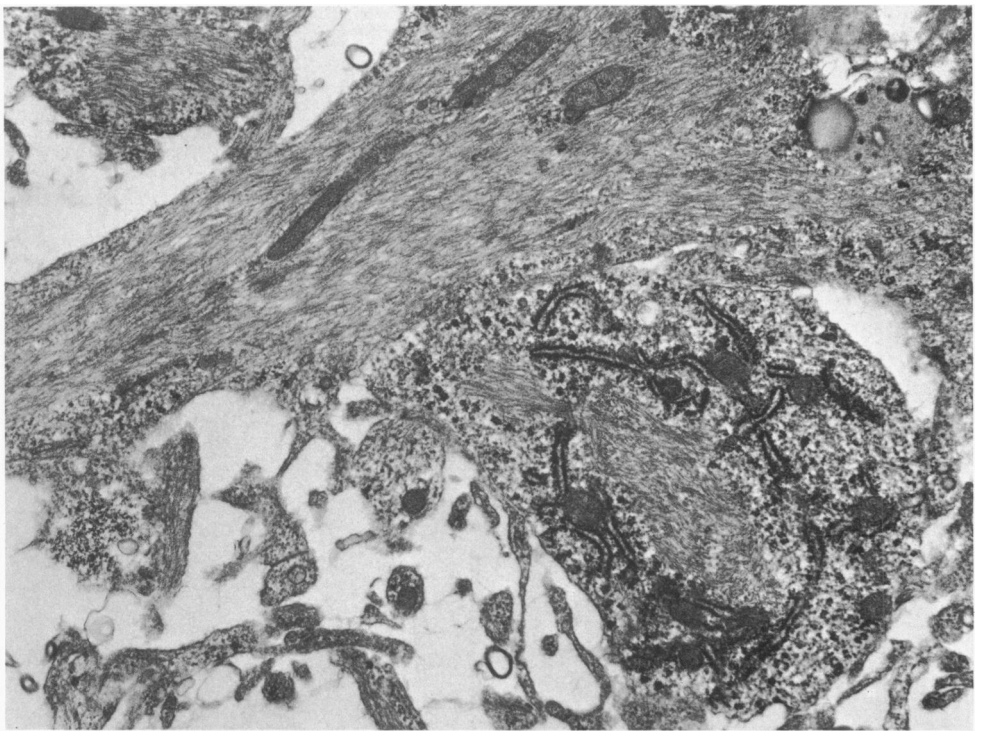
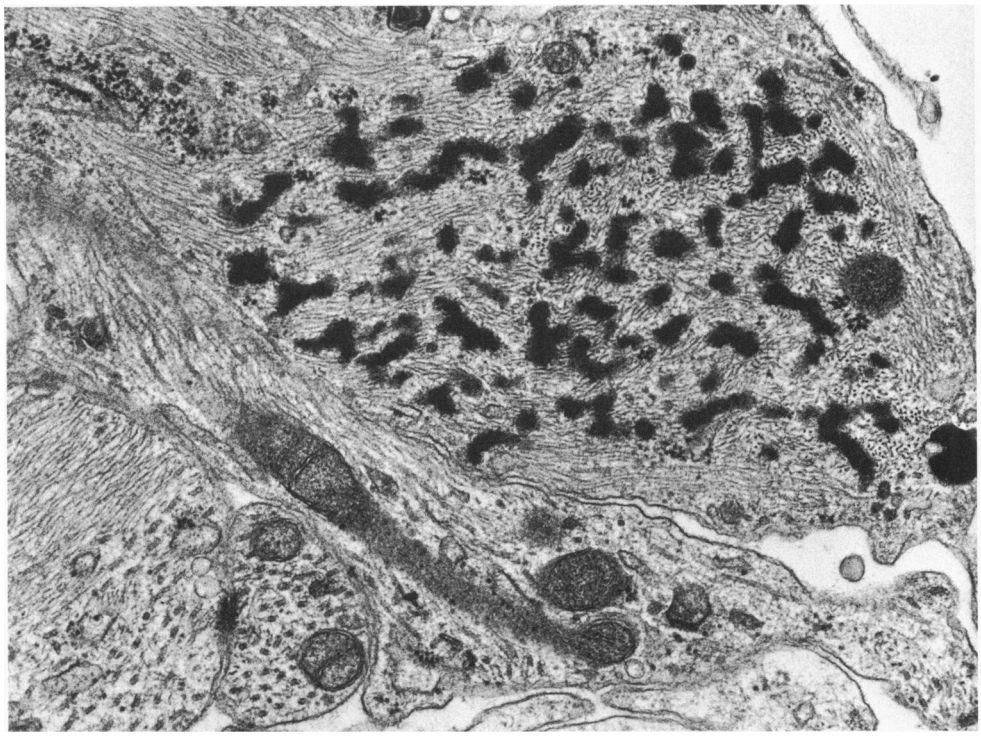


Fig 7—Cerebellar astrocytoma cultured on sponge foam, 8 days *in vitro*. Tumor cell with uniform oval nucleus, several collections of Golgi apparatus, lysosomes and loosely arranged glial filaments. Tumor cell process (*right upper corner*) contains more compact filaments (× 13,300). **Fig 8**—Cerebellar astrocytoma on sponge foam, 29 days *in vitro*. Intimate association of increased granular endoplasmic reticulum, free ribosomes, and glial filaments packed in broad bundles in tumor cell processes (× 15,000).



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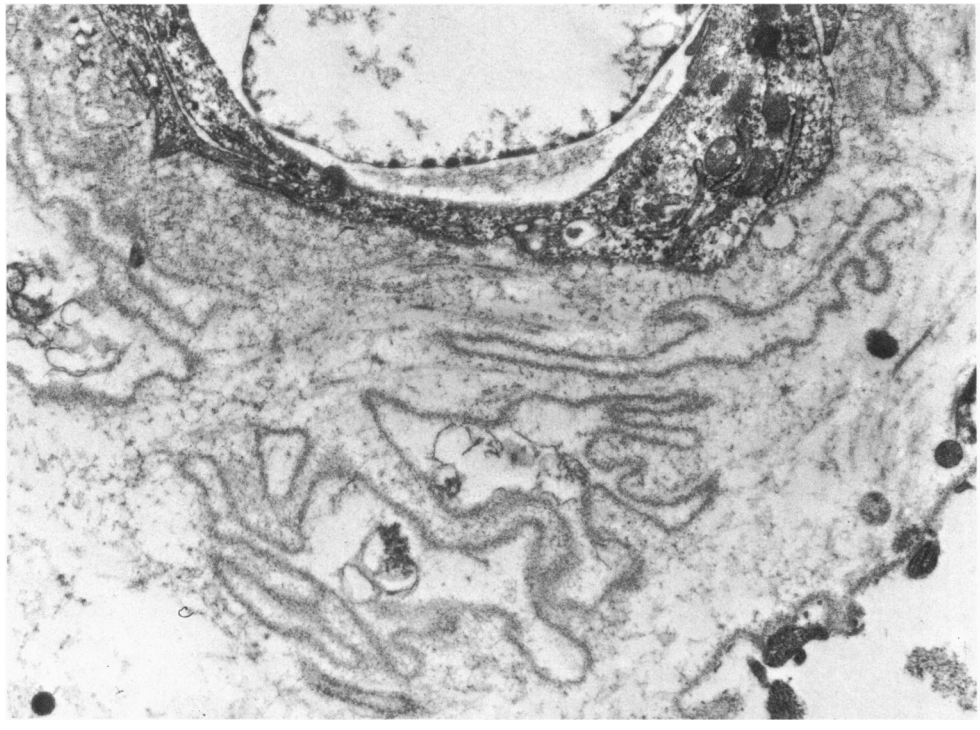

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Fig 9—Cerebellar astrocytoma on Millipore filter, 15 days *in vitro*. One tumor cell process contains numerous densely osmiophilic bodies which merge with adjacent glial filaments. This corresponds to a Rosenthal fiber. Three other processes (*lower left and center*) contain microtubules and glial filaments; two of these processes are joined by a punctum adhaerens (× 53,300). **Fig 10**—Cerebellar astrocytoma on sponge foam, 43 days *in vitro*. Pericapillary basement membrane material demonstrating elaborate folding and reduplication (× 14,600).

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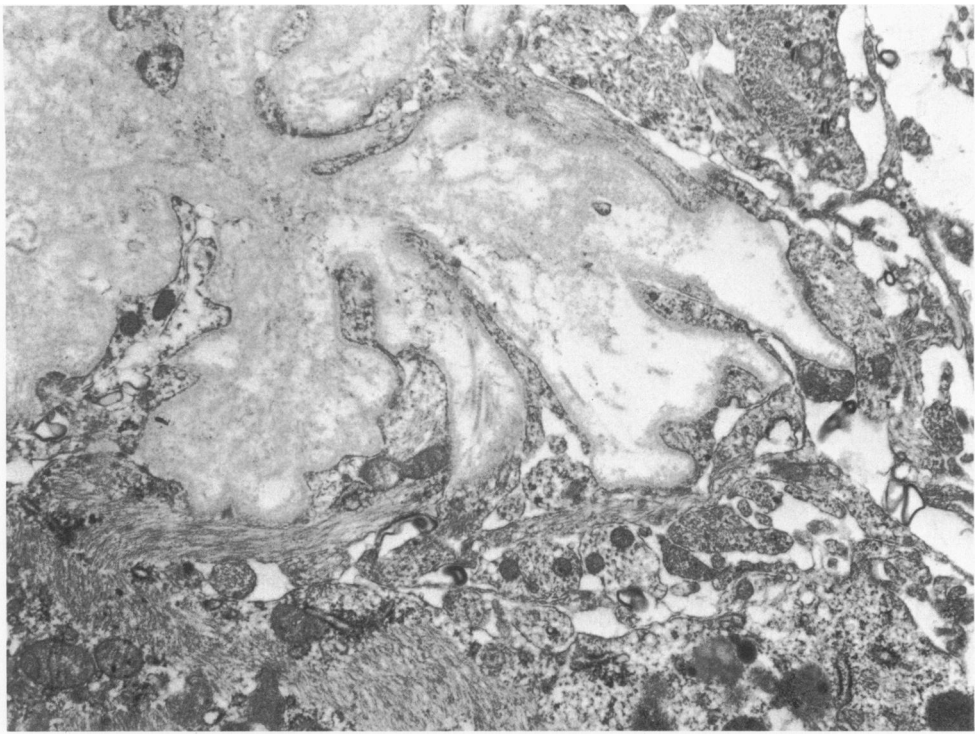

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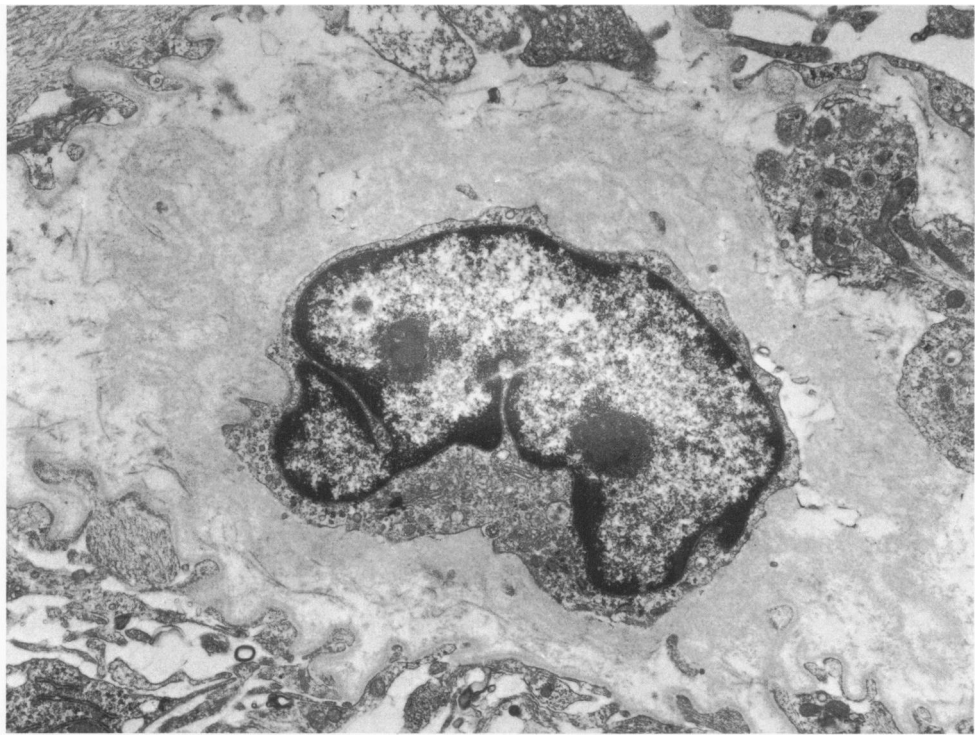

Fig 11—Cerebellar astrocytoma on sponge foam, 29 days *in vitro*. Outer border of increased basement membrane material illustrating its apposition to tumor cell processes which contain abundant glial filaments (× 14,200). **Fig 12**—Same culture as Figure 11. Isolated cell, probably mesenchymal, surrounded by excessive basement membrane material. Numerous filament-containing tumor cell processes are closely apposed to the outer discrete border of the basement membrane material (× 9300).