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Invasion of experimental rat brain tumor: early morphological changes following microinjection of C6 glioma cells

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Summary. We present morphological data of the early stage of tumor invasion in the central nervous system. C6 rat glioma cells were injected into the caudate-putamen of rat brain using glass micropipettes to minimize traumatic reactions. Four days after the inoculation, we examined the tumor-brain interface using light and electron microscopy. Ultrastructurally the tumor processes were attached to the perivascular basement membrane instead of the astroglial end-feet. At the tumor periphery, the vessel walls were in contact with both tumor processes and astroglial end-feet. Astrocytes withdrew their processes from the vascular walls and changed into a reactive phenotype, while the neuronal cells remained virtually intact, even when surrounded by tumor cells. Immunohistochemical study using C6 cells labeled with bromodeoxyuridine showed migration of the cells toward the perviascular space that was distant from the site of injection. These observations represent the earliest morphologically detectable changes of the tumor-brain interface, and suggest that the C6 cells possess the characteristics of high affinity to the endothelial basement membrane and invade along the preexisting blood vessels with brain parenchymal infiltration.

Key words: C6 glioma – invasion – ultrastructure – immunohistochemistry

Maintenance of the territorial integrity of the brain, including the neurons, glial cells, blood vessels, and meninges, is under strict regulatory control during embryogenesis as well as in adult life. When a malignant brain tumor develops, its growth is regulated not only by intrinsic tumor cell kinetics, but also by the interaction of tumor cells with normal and reactive cells in the tumor environment [30]. One of the most lethal properties of malignant cells is their ability to infiltrate normal tissue and to metastasize to distant sites; thus invasion is the hallmark of the malignancy [21].

Carcinogenesis is usually a multistage process that involves the activation, mutation, or loss of different genes. In the affected cells these changes result in loss of growth control, attraction of blood vessels, and invasion of neighboring tissue [16, 24]. Glial tumors are typically angiogenic and infiltrate surrounding brain parenchyma. These mechanisms probably differ from those of systemic neoplasms due to the fact that primary central nervous system (CNS) tumors rarely metastasize systemically [8]. Even the most aggressive brain metastases tend to form noninfiltrative masses with well-defined borders [22]. Glial tumor cells degrade large extracellular macromolecules and transgress normal barriers such as basement membrane [30]; thus, the investigation of tumor-brain interface may be of great importance in understanding the tumor invasion.

Three experimental methods are commonly used to induce cerebral tumorigenesis: radiation, viruses, and chemical carcinogens [13]. Such methods do not easily offer an opportunity to create identical tumors in similar locations in all experimental animals [1]. Although inflammatory or traumatic irritation may develop, the implantation of a carcinogen offers the advantage of producing the same tumor in the same region at any stage of tumor development, and it has been used for a number of cell lines [1, 11, 12].

Astrocytoma C6 is a well established in vitro cell line initially induced in rats by *N*-nitroso-methylurea [2], and extensively characterized thereafter [6, 26]. Implantation of C6 glioma into the rat brain mimics many of the growth and pathological characteristics of human gliomas [35]. In this study, we investigated the early pathological changes of tumor cell invasion, injecting C6 glioma cells using micromethods in vivo. To reduce the incidence of traumatic or inflammatory reactions, we used a glass micropipette to implant tumor cells into the brain originally used in horseradish peroxidase (HRP) microinjection [31]. At the early stage of invasion, the

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tumor cells attached preferentially to the perivascular basement membrane instead of the astroglial end-feet and spread along the perivascular space. Astrocytes changed into a reactive phenotype, while neurons exhibited little ultrastructurally detectable changes in vivo.

Materials and methods

Cell culture

Rat C6 glioma cell line originally cloned from an *N*-nitroso-methylurea-induced glioma by Benda et al. [4] was obtained from the American Type Culture Collection (Rockville, Md.) and maintained in monolayers in 100-mm dishes (Corning, Iwaki, Japan) at 37 °C under humidified 5 % CO_2-95 % air. The cells were cultured in Eagle's minimum essential medium (MEM, Gibco, Grand Island, N.Y.) supplemented with fetal bovine serum (FBS, 10 % final concentration, Biocell, lot 6201, Calif.), penicillin-G (50 unit/ml), and streptomycin (50 μg/ml). Cells were harvested during the log phase of growth.

Rat skin fibroblasts were used as controls. The skin was cut into small pieces in MEM under sterile conditions, attached to 35-mm collagen-coated plastic dishes (Corning 25000COL1), and cultivated in MEM supplemented with 10 % FBS. The medium was changed every 3 days until cell growth was confluent. The cultures were subsequently subcultured at a 1:2 split ratio. Cells were used after the third or fourth passage.

Animals and microinjection of tumor cells

Eighteen adult male Wistar rats weighing between 250–300 g were used. They were anesthetized with sodium pentobarbital (Nembutal, 100 mg/kg) during surgery and injection. Each animal was securely placed in a stereotactic surgical frame. A glass micropipette was inserted into the right caudate-putamen to a depth of 4.5 mm. The diameter of the tip of the micropipette was 25–30 μm. Cell suspension of C6 glioma cells ($n = 12$) or rat skin fibroblasts ($n = 3$) (5 μl of MEM supplemented with 10 % FBS containing 10^5 cells) was injected under a pressure of 2.0–3.0 kg/cm^2 for 1–2 min. Control rats were injected with 5 μl of MEM supplemented with 10 % FBS alone. The craniectomy was sealed with bone wax and the overlying skin incision was closed.

Ultrastructural observation of early neoplastic lesion

The animals were killed 4 days after the injection. They were anesthetized with sodium pentobarbital and perfused with 300 ml Ringer's solution, followed by 400 ml of a fixative containing 2 % glutaraldehyde and 3 % paraformaldehyde, in 0.1 M phosphate buffer (pH 7.4–7.6), at room temperature. The brain was then removed from the cranial cavity and serial coronal sections of the cerebral hemispheres were cut at 200 μm. Tissues were fixed with the same fixative as used for perfusion for 2 h at room temperature, and postfixed with 0.5 % OsO_4 buffered with a acetate-veronal solution (pH 7.5) for 30 min at 4 °C. Osmicated tissues were stained with 0.5 % uranyl acetate overnight, dehydrated in graded ethanol, and embedded in Epon 812. Flat embedding molds were used to faciliate the proper orientation of the specimens for subsequent ultramicrotomy. Semithin sections (1 μm thick) of tissues at the injection site were stained with 1 % toluidine blue in 1 % borax. Ultrathin sections which were trimmed precisely to investigate the interface between the tumor and the normal brain were cut on a Reichert-Jung Ultracut E (Wien, Austria), contrasted with uranyl acetate and lead citrate, and examined with a JEM-100B-TR electron microscope (JEOL, Tokyo, Japan).

Immunohistochemistry

In some experiments ($n = 3$), we injected C6 tumor cells which had been treated with 5- bromo- 2′-deoxyuridine (BrdUrd) to determine the distance of the tumor cell migration from the site of injection in 4 days. Tumor cells were cultured in the medium containing 10 μg/ml of BrdUrd for 24 h. The BrdUrd-containing medium was then removed, and the cells were washed twice with phosphate-buffered saline to ensure the complete removal of unbound BrdUrd. The cells were trypsinized and suspended in MEM supplemented with 10 % FBS, and injected into the caudate-putamen using the method described above. After 4 days the animals were killed and perfused with 4 % paraformaldehyde solution. The cerebral hemispheres were embedded in paraffin and the slices (5 μm) were made. The tumor cells that had incorporated BrdUrd were identified by an immunoperoxidase technique (Cell Proliferation Kit; Amersham, Tokyo, Japan). Tumor-bearing slices were also stained immunohistochemically for glial fibrillary acidic (GFA) protein according to the peroxidase-antiperoxidase method (Dako, Tokyo, Japan). Antisera against GFA protein were obtained from Collaborative Research (Two Oak Park, Bedford, Mass.).

Results

C6 tumor cell microinjection

The procedure for implanting an intracerebral tumor was well tolerated; the incidence of tumor development was nearly 100 %. When the rats with the implanted tumor were killed at 7 or 14 days following microinjection, the tumor had grown too large to detect the tumor-brain interface precisely. Rats appeared clinically normal for the 4 days of observation after the implantation. The site of implantation was not grossly visible when the brain was removed from the skull. There was no extension of the tumor extracerebrally into the subarachnoid or subdural spaces in any animal. Examination of the toluidine blue-stained sections revealed that the tumor grew as an undifferentiated astrocytoma and demonstrated histological malignancy; mitotic fig-

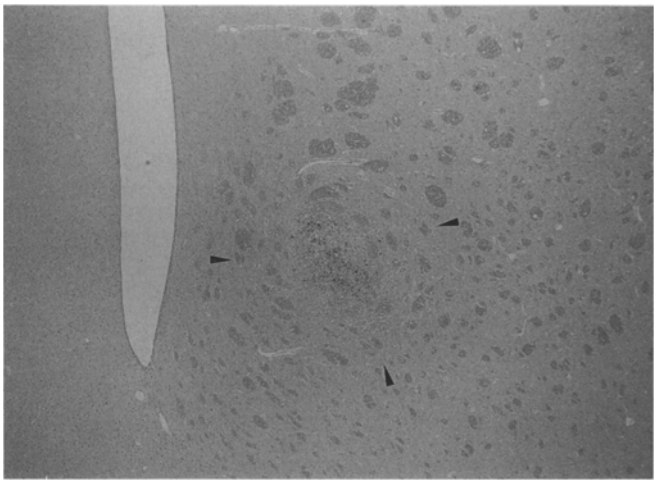


Fig. 1. A transplanted tumor is located in the right caudate-putamen (*arrowheads*). No micropipette tracks are seen. Hematoxylin staining, × 20

ures, hypercellularity, and anaplasia. The tumors were generally demarcated and their diameters ranged from 100 to 200 μm. No micropipette tracks were found in thick sections (Fig. 1). The perivascular space around the injected site was not enlarged, and the edematous zone was limited.

Ultrastructural findings

Neoplastic cells. The tumor-brain interface was studied by transmission electron microscopy. In the center of the tumor, neoplastic cells showed filiform protrusions which built up connections between the cells by crossing

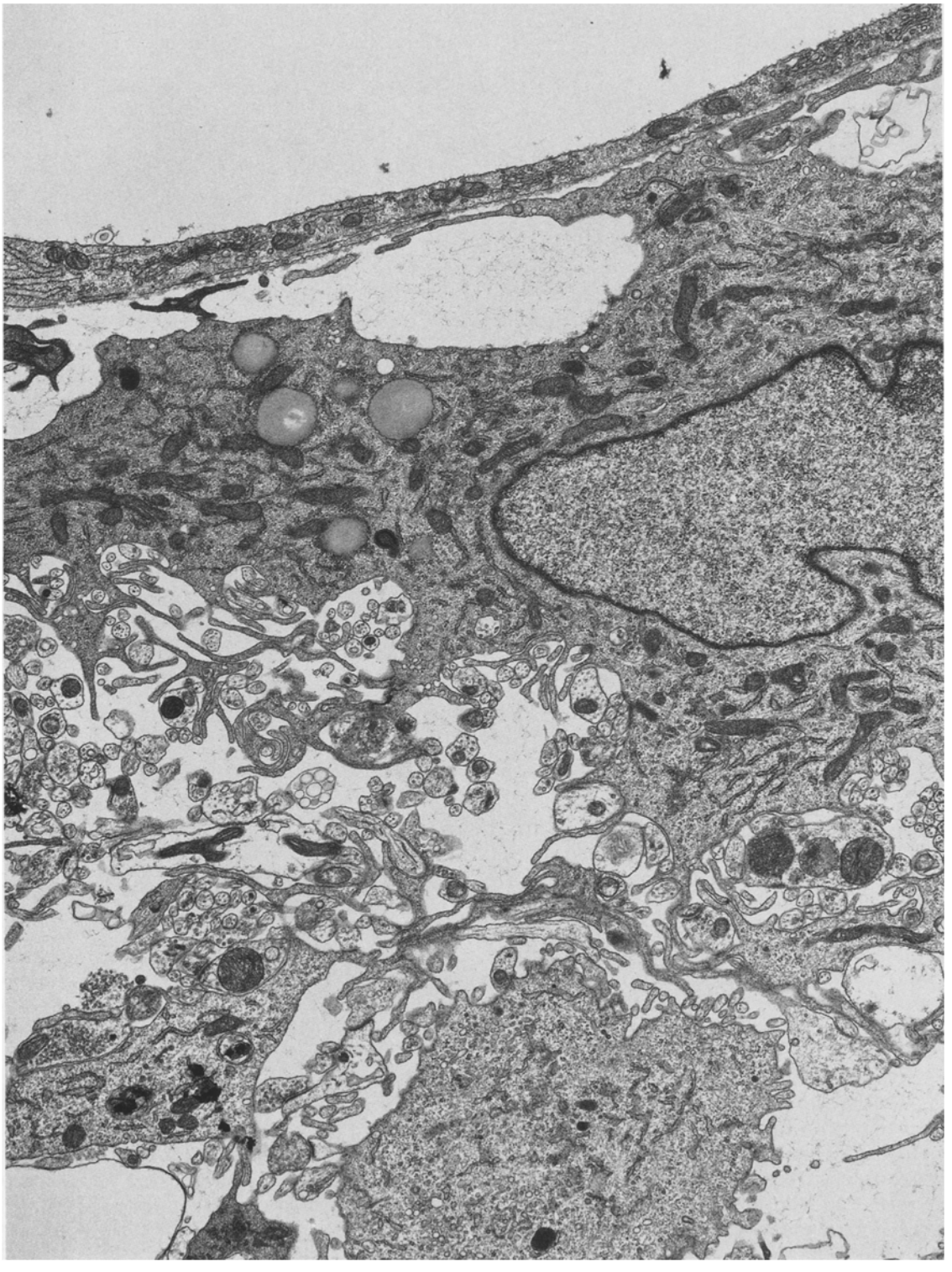


Fig. 2. Electron micrograph of the center of the transplanted tumor. Neoplastic cells have multiform nuclei, well-developed Golgi apparatus, many mitochondrias, and a number of polysomes. Extracellular space is widened and filled with fine amorphous material compared to the periphery of the tumor. × 3050

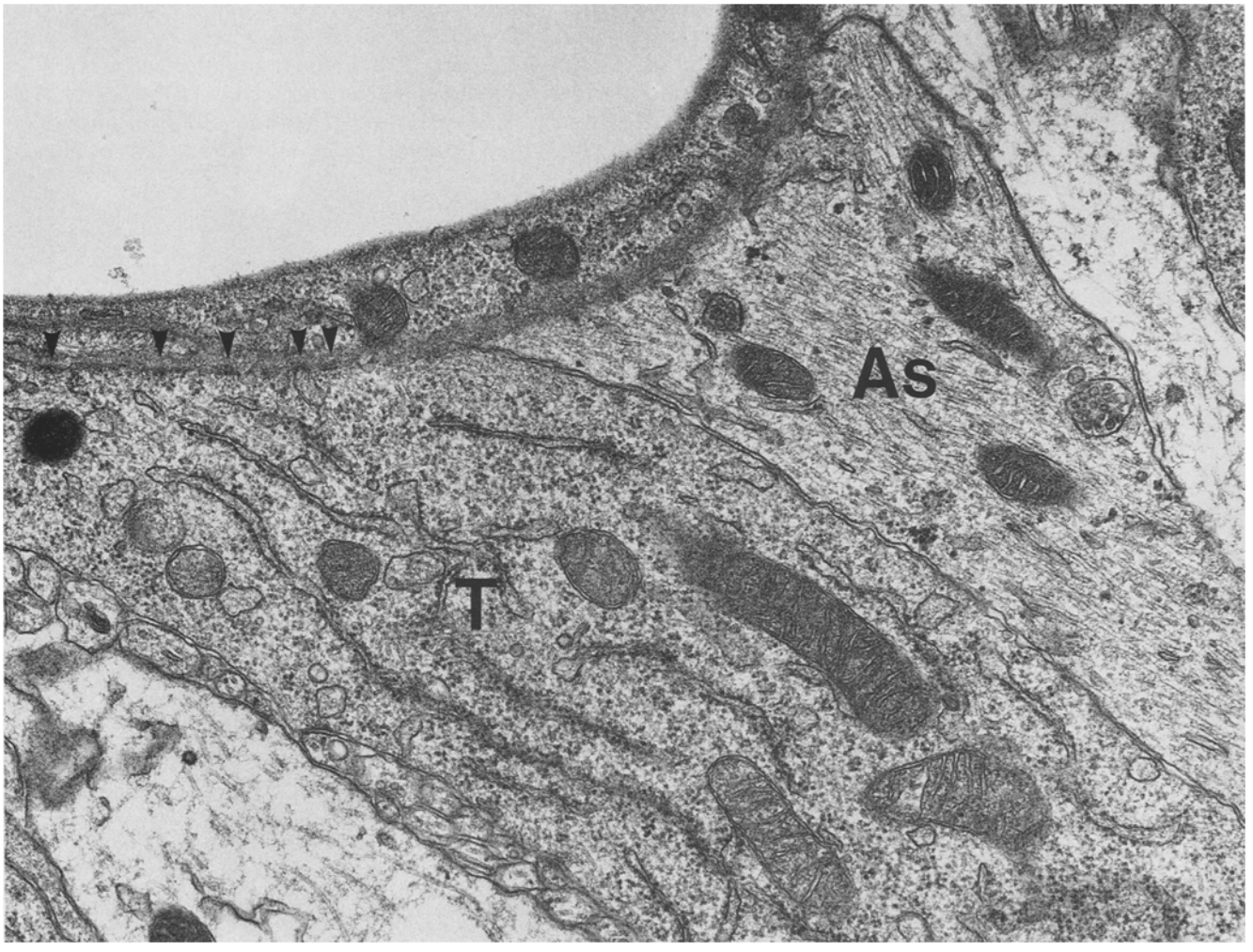


Fig. 3. Electron micrographic view of the vessel wall at the tumor periphery. Massive processes of the neoplastic cell (*T*) attached to the perivascular basement membrane, and co-exist with astroglial end-feet (*As*) including bundles of glial filaments. The interface between tumor process and endothelial basement membrane had electron-dense plaques underlayered by a subbasal dense plate (*arrowheads*). × 7500

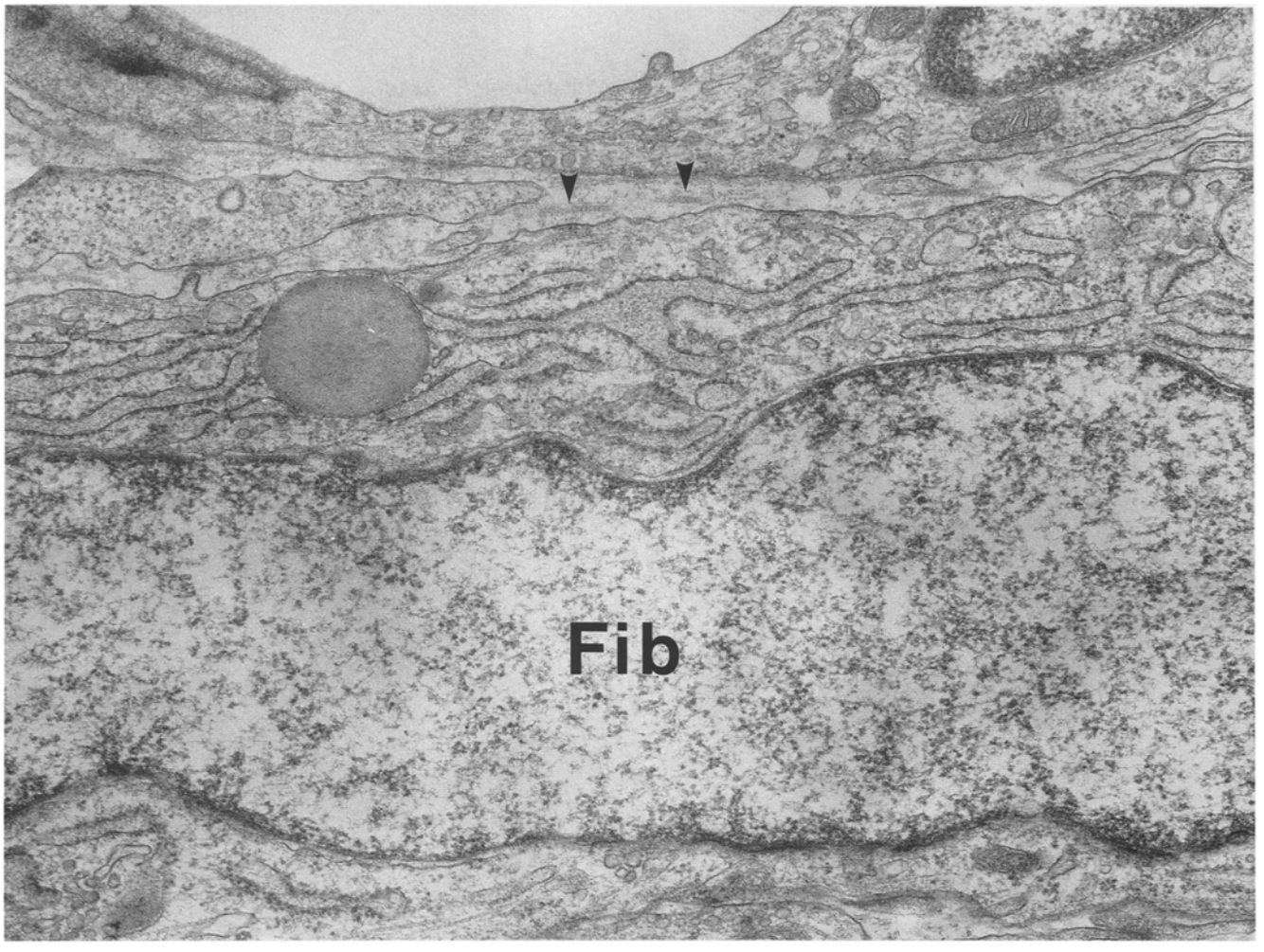
an enlarged extracellular space (Fig. 2). The polymorphic nuclei were mainly located in the center of the tumor cell. Chromatin was accumulated near the nuclear membrane and the nucleolus. Mitochondria were distributed throughout the cytoplasm and exhibited regular cristae and a dense matrix. There were multiple Golgi complexes, rough endoplasmic reticulum, many free ribosomes and intracellular digestive apparatus. Occasional microtubules were interspersed with filaments. These ultrastructural findings could differentiate neoplastic cells from reactive astrocytes that had characteristic glial filaments. At the periphery of the tumor, neoplastic cells were seen infiltrating the brain tissue with several cellular protrusions. Some of them were attached to the perivascular basement membrane instead of the astroglial end-feet. Hemidesmosomal plaques were observed along the cell membrane in the neoplastic processes attached to the endothelium (Fig. 3).

In control experiments, we injected rat skin fibroblasts into rat caudate-putamen using the method described. Ultrastructurally, the fibroblasts demonstrated few protrusions and did not attach to the perivascular basement membrane (Fig. 4). Collagen fibers were observed in the extracellular space.

Reactions of host cells. In the tumor center, the extracellular space was widened and filled with fine amorphous material (Fig. 2). The vessels were composed of

Fig. 4. Rat skin fibroblasts are injected into the caudate-putamen. The fibroblast (*Fib*) has few protrusions and does not attach to the perivascular basement membrane. Note that some collagen fibers are present in the extracellular space (*arrowheads*). × 9000

Fig. 5. Electron micrograph showing a reactive astrocyte at the tumor periphery. The cell exhibits well-developed Golgi apparatus and neumerous mitochondria. An excessive number of glial filaments, which are irregularly and abruptly assembled, are present in the cytoplasm. × 7500



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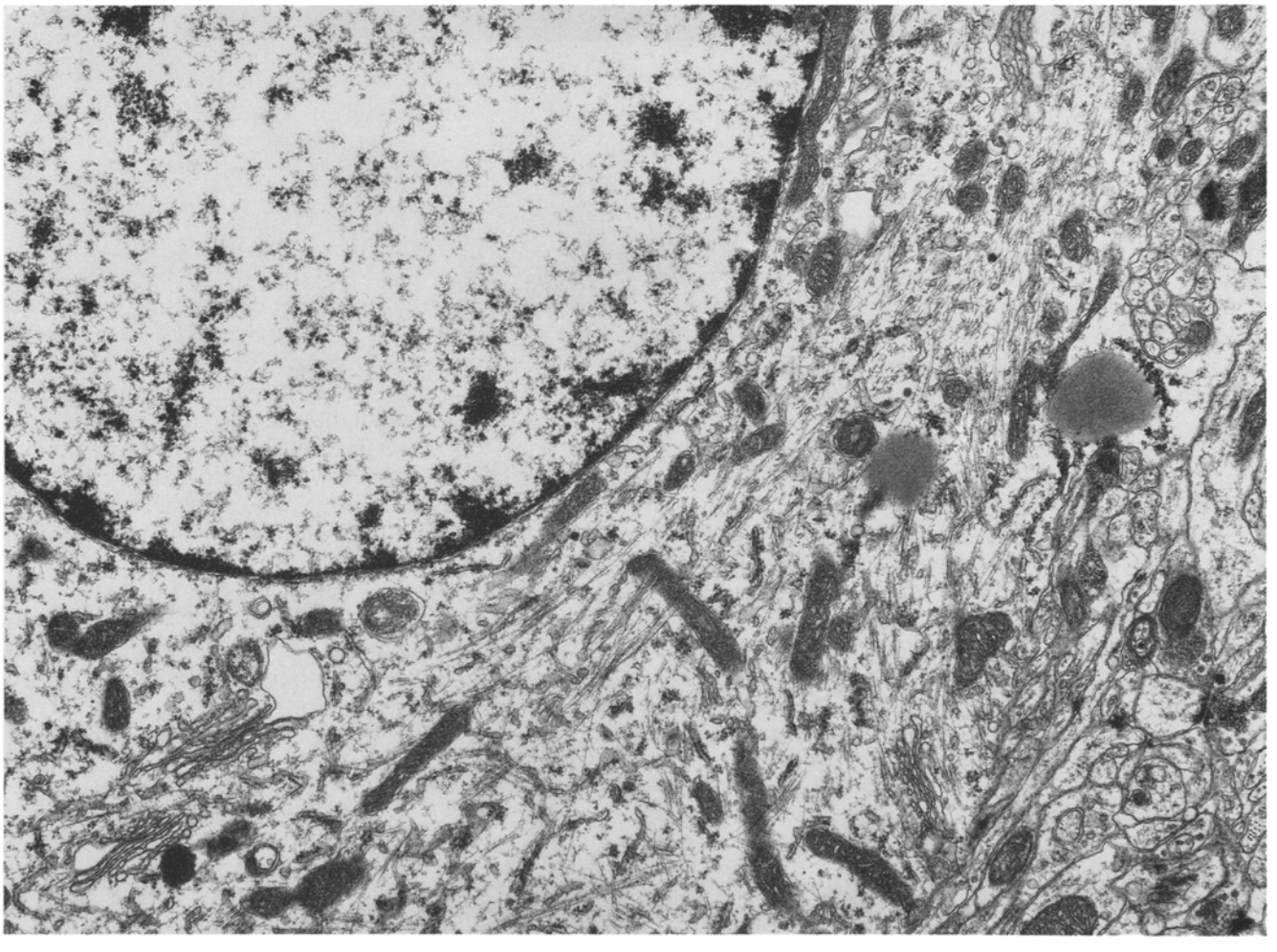

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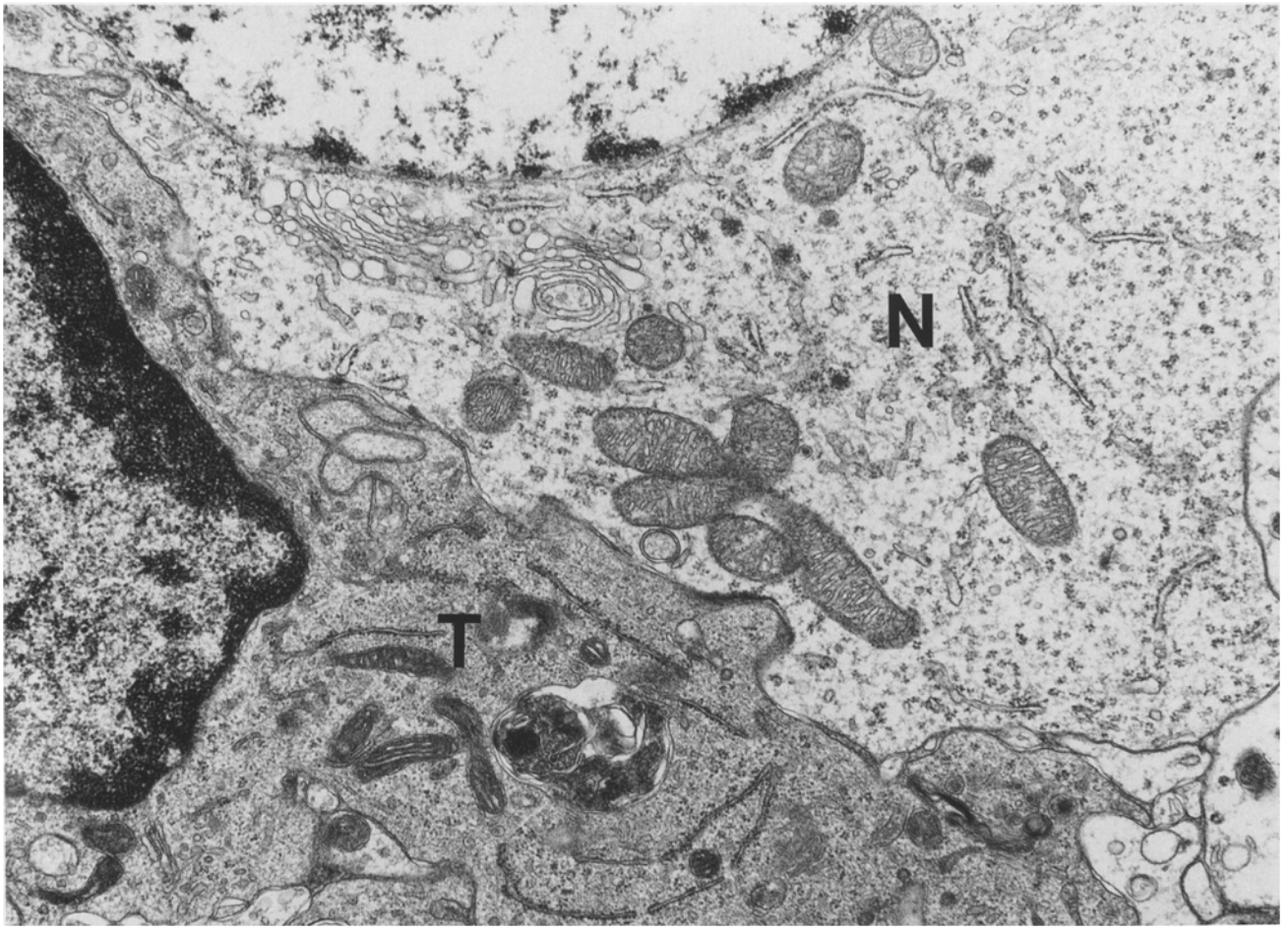

Fig. 6. Electron micrograph of a neuronal cell (*N*) that is immediately contact with a neoplastic cell (*T*). The neuron shows no destructive changes at this early stage of invasion. × 7500

one-layered and flattened endothelium. Endothelial proliferation was not found around the transplanted area at the early stage of tumor invasion. An important ultrastructural observation was that the preexisting vessels were deprived of the glial sheath and surrounded by neoplastic processes. At the tumor periphery, astroglial foot processes including glial filaments co-existed with neoplastic protrusions around the perivascular basement membrane (Fig. 3).

Reactive astrocytes were present around the tumor (Fig. 5). These cells were large and irregular with cell processes, an eccentric nucleus, and various cell organelles. An excessive number of glial filaments were observed in the cytoplasm. As compared with the intact astrocytes, the filaments were irregularly and abruptly assembled.

The axons and myelin sheaths of the white matter were degenerated due to the invasive growth of tumor cells. However, the neuronal cells remained virtually intact, even when they were in direct contact with the tumor cells at the early stage of invasion (Fig. 6).

Immunohistochemistry

We investigated the movement of the tumor cells in vivo using an immunohistochemical method. The tumor cells which incorporated BrdUrd into their cellular DNA were identified using an anti-BrdUrd monoclonal antibody. Although most of the labeled nuclei of a tumor cell were closely packed near the periphery of the tumor, some of the labeled nuclei were found in the perivascular space away from the primary site of injection (Fig. 7). We interpreted this finding as the migration of tumor cells along the perivascular space.

Paraffin-embedded sections were also stained using GFA protein antisera. A frame network of delicate processes and the presence of swollen cytoplasm in the astrocytes were observed around the tumor (Fig. 8). The nuclei were not stained, and perinuclear accumulation was seldom seen. Control sections of rat normal brain showed only faint perivascular staining of astrocytic processes. C6 glioma cells showed negative staining for GFA protein.

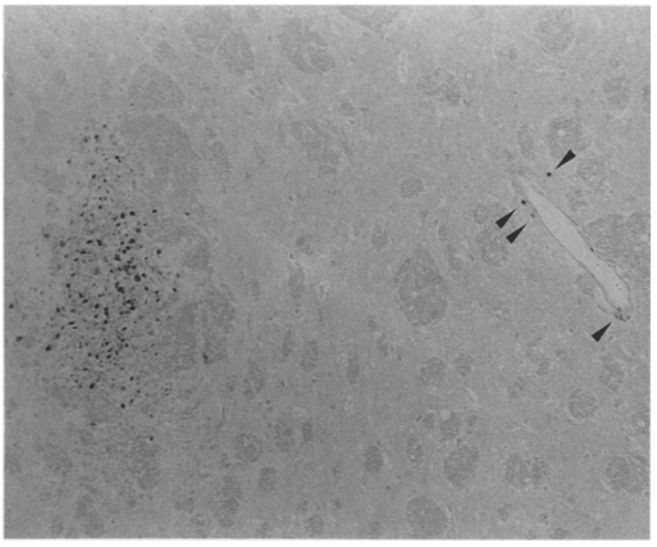

Fig. 7. Identification of neoplastic cells that incorporated BrdUrd into their cellular DNA using anti-BrdUrd monoclonal antibody. Although the labeled tumor cells are closely packed, some are found at the perivascular space near the site of injection (*arrowheads*). × 54

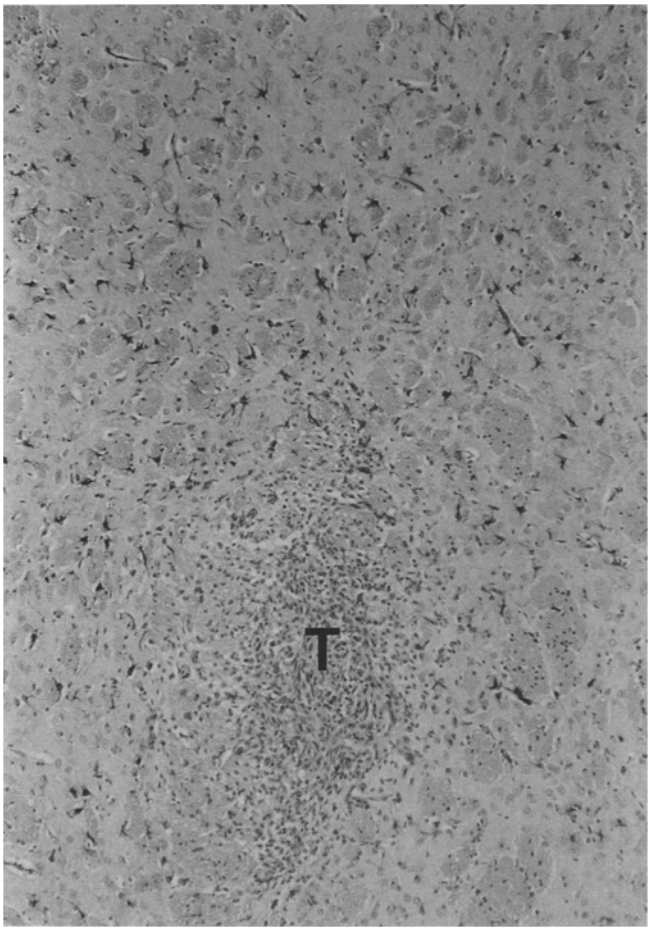

Fig. 8. GFA protein-positive cytoplasm and frame network of processes are observed around the transplanted tumor (*T*). The neoplastic cells are negative for GFA protein. × 70

Discussion

Invasion is a determinant of tumor malignancy since it leads to the death of the host either directly or indirectly. Tumor invasion involves a series of pathological interactions between the malignant cells and host stroma [14, 27]. Glial tumors display a high level of infiltration to surrounding brain parenchyma, although the rate of cell proliferation is less than that of tumors derived from organs other than the brain. The invasive nature of glial tumors makes attempts at treatment most difficult, and defines them as one of the most malignant tumors among the neoplasms [9]. Early studies have suggested that the preneoplastic or precancerous lesions are crucially important in the understanding of tumor development [17, 33]. Investigations of the interface between the tumor and normal brain may provide a clue to understanding the mechanisms of tumor invasion.

Although the morphology of the experimental gliomas has been studied extensively [15, 17], little is known about the early neoplastic changes. An animal model of brain tumor should effectively mimic the characteristics of human malignant gliomas both developmentally and within the clinical context [11]. Chemical-, viral-, and radiation-induced tumos are less than ideal since their latency is not predictable or they induce a variety of tumor types [11]. The intracerebral injection technique of tumor cells is thought to be a satisfactory method for reproducible tumor production. Transplanted astrocytomas, however, have some disadvantages compared with other experimental brain tumors, in that one cannot avoid the production of traumatic or inflammatory irritation. In this study, we used glass micropipettes originally used for microinjection of HRP into astrocyte [31]. Its tip diameter was 25–30 μm. When the tumor cells were injected with a 26-gage needle, edematous changes were observed around the site of injection, and the capillary walls were deprived of contact with the glial processes as if the vessels seemed to float in a floccular extracellular space (data not shown). Using a glass micropipette, no needle tracks were found, and the astrocytic foot processes were well preserved around the vessels near the site of injection.

Electron microscopic analysis revealed that the tumor cells had a well-developed Golgi apparatus, rough endoplasmic reticulum, and many free ribosomes, suggesting an activated metabolism. Like other neoplastic astrocytes [23, 34], we observed that the C6 glioma cell produced microvilli and short protrusions that contacted the perivascular basement membranes. At the periphery of the tumor, tumor processes co-existed with the astroglial end-feet around the vessel walls. No such findings were seen when the skin fibroblasts were injected into the rat brain. Similarly 9L gliosarcoma, which has lost ability to infiltrate, has been shown to invade primarily by growing along pre-existing blood vessels in Virchow-Robin spaces without infiltrating the brain parenchyma, and without forming large, solid masses [3]. C6 glioma cells appeared to invade along the perivascular space with their processes attached to the endothelial basement membrane instead of the astro-

glial end-feet. This mode of invasion might be specific to C6 glioma cells that possess the characteristic of high affinity to the endothelial basement membrane, which has been seen with astrocytes. At the interface between tumor processes and basement membrane, electron-dense plaques were seen that were underlayered by a subbasal dense plate (Fig. 3). Carter et al. [7] have shown that several integrin complexes, which are associated with hemidesmosomes, are involved in cell-extracellular matrix attachment. It is likely that these hemidesmosomal plaques may play a role in the attachment of neoplastic cells to the basement membrane.

Proliferation and hypertrophy of astrocytes are major cellular reactions associated not only with injury to the central nervous system but also with tumor growth [10, 19, 28]. Astrocytic proliferation may be in part beneficial, because the cells produce trophic factors that promote survival of the neuron and neuritic growth [20], and secrete components of basal lamina that may guide regenerating axons [5]. It was also shown that C6 glioma-conditioned medium can promote the survival and outgrowth of neurites [2]. In our observations the neuronal cells, even when surrounded by the neoplastic cells, showed little destructive changes in the early stage of invasion. This might be due to the trophic factors secreted by the reactive astrocytes or by the tumor cells themselves.

The immunohistochemical study using C6 cells labeled with BrdUrd showed migration of the tumor cells toward the perivascular space that was distant from the primary site of injection. Cultured glioma cells have been shown to secrete enzymatically active proteases that may contribute to the infiltrative behavior of glial tumors [18, 29]. Recently, Schwartz et al. [32] have suggested that the infiltrative ability of C6 glioma cell is controlled on the individual cell level, including membrane-associated proteases [25] and cell motility [21]. Our observations also indicate an increased motility of C6 cells and a tendency of the cells to migrate toward perivascular space. This mode of infiltration may be one of possible mechanisms of early invasion in human glioma cells. However, there is no evidence that this is a predominant mode of invasion in human gliomas, probably because of the limitation of available human brain tumor materials.

Although previous reports have provided some information on tumor invasion, the present study is the first to carry out sequential ultrastructural examinations of the tumor-brain interface in vivo. The tumor cells invaded through the perivascular space with its processes attached to the vascular walls instaed of the astroglial end-feet. Astrocytes withdrew their foot processes from the perivascular basement membrane and developed into a reactive phenotype, while the neuronal cells showed little destructive changes in this condition. These findings represented the earliest morphologically detectable changes of brain tumor invasion in the CNS.

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