

Histopathology, Invasion, Migration and Tumorigenicity in the C6 Rat Glioma Model

C6 Rat Glioma Modelinde Histopatoloji, Invazyon, Migrasyon ve Tümör Gelişimi

ABSTRACT

Glial tumors are the largest group of central nervous system tumors and glioblastoma multiforme is the most common form. Glioblastomas are anaplastic forms of gliomas. Their high incidence and malignity have led researchers to work hard to better understand and treat these tumors. It is now known that genetic factors play a role in glioma etiopathogenesis. Of the differences in genetic built, two attract the most attention: oncogenes that lead to cell division and the occurrence of cells that have lost tumor suppressor genes. After realizing that the evolution of surgery and radiotherapy would have no significant value for malignant brain tumors, the trial of potential chemotherapeutic, genetic and immunologic therapy methods in an appropriate experimental glioma model has gained in importance. The C6 rat glioma model is used in these studies because of its similarity to the human glioblastoma. In this study, we used the C6 rat glioma model which has glioblastoma-like effects and tried to understand these tumors better by detecting their tumorigenicity, invasion and migration characteristics in the research laboratory of our clinic.

KEY WORDS: C6 rat glioma, migration, invasion, histopathology

ÖZ

Glial tümörler santral sinir sisteminin tümörlerinin en büyük grubudur. Glial tümörlerin içerisinde en sık görüleni glioblastoma multiformedir. Glioblastomalar gliomaların en anaplastik şeklidir. Bu tümörlerin görülme insidansındaki yükseklik ve maligniteleri araştırmacıları bu tümörleri daha iyi kavrama ve tedavi edebilme yolunda yoğun çaba harcamaya teşvik etmiştir. Glioma etiopatogenezinde genetik faktörlerin önemli bir yer tuttuğu artık bilinmektedir. Genetik yapıya ait değişikliklerden iki tanesi ön plandadır. Hücre bölünmesini ortaya çıkaran onkojenlerin varlığı ve tümör süpressör genlerini kaybetmiş hücrelerin ortaya çıkması. Malign beyin tümörlerinde cerrahi ve radyoterapi metodlarının daha da geliştirilmesinin, bu tümörlerin prognozu açısından anlamlı faydası olamayacağı şeklindeki tespitin ardından; uygun deneysel glioma modeli üzerindeki potansiyel kemoterapötik, genetik, immünolojik tedavi metodlarının denenmesi gündeme gelmiştir. C6 rat glial tümör modeli insan glioblastomasına benzerliğinden dolayı bu araştırmalarda kullanılmaktadır. Hastanemiz araştırma laboratuvarında gerçekleştirdiğimiz bu çalışmada; glioblastoma benzeri etki gösteren C6 rat glial tümör modeli oluşturarak tümör gelişimini, invazyon ve migrasyon tarzındaki davranışlarını tespit ederek bu tümörleri daha iyi anlamaya çalıştık.

ANAHTAR KELİMELER: C6 rat glioma, migrasyon, invazyon, histopatoloji

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INTRODUCTION

Glial tumors comprise the largest group of the central nervous system tumors (45-55% in hospital series). Their average incidence is 4/100.000 (7). The most common glial tumor is glioblastoma multiforme. Glioblastomas are the most anaplastic type of gliomas. Their high incidence and malignancy have led the researchers to strive to better understand and heal these tumors. In fact, it is hard to classify gliomas because of the complexity of their histopathology (18,25). They do not fit in the criteria and classification properties of other tumors as they localize in an important organ like the brain. A glial tumor that is histologically benign can be clinically malignant because of its location (14,19). It is therefore difficult to group gliomas according to their clinical properties, morphology or localization. Various glioma classification systems have been suggested. Tooth in 1912 noticed the relationship between prognosis and histological appearance in gliomas that differ in basic criteria like location, histopathology, clinical course, therapy alternatives and prognosis. This was an innovative approach to glial tumor classification (20). In 1926, Bailey and Cushing proposed a classification based on the morphological stages that central nervous system cells pass during embryogenesis. This classification has been the basis of many classifications that are used today (10,18). In 1949, Kernohan et al. proposed a classification based on the degree of anaplasia of the cells that are produced by gliomas. In 1988, Daumas-Duport proposed a prognostic classification based on nuclear atypia, mitosis, endothelial proliferation and necrosis. The WHO (World Health Organization) forever changed the way we look at brain neoplasms when their landmark classification was published in 1993. In 2000, a further refinement of this classification system was published. The WHO classification classifies neoplasms by their overall biologic potential. Lower grade tumors have the best prognosis. The category of Grade 1 is reserved for neoplasms that have a stable histology. Tumors that appear histologically "benign", yet are known to progressively transform over time, are not classified as Grade 1 lesions. The WHO classification also recognized and defined some new specific subtypes of astrocytoma like gliosarcoma and giant cell glioblastoma. These subtypes were also assigned a grade.

Glioblastomas are highly cellular and highly anaplastic tumors. Infiltrative growth, necrosis, foci of hemorrhage, endothelial proliferation in vessel walls, invasion and migration to distant subarachnoid regions are some microscopic features. It is now well-known that genetic factors play an important role in glioma etiopathogenesis (2,15,17,18).

Of the differences in genetic built, two attract the most attention:

1-The presence of oncogenes that provoke cell division

2-The occurrence of cells that have lost tumor suppressor genes

Tumor suppressor genes are located at the 10th and 17th chromosomes in astrocytomas (18,30).

Malignant astrocytomas are significant stimulators of angiogenesis in the physiologic environment while angiogenesis plays an important role in the development of astroglial tumors. This abnormal development is thought to be related to cell genetics (7). Cranial irradiation and some chemicals may disturb cell genetics by their mutagenic effect and cause astrocytoma formation. Also the weak immune response of the organism to these tumors facilitates tumor formation (18,22).

Despite all the efforts of neurosurgeons, oncologists, radiotherapists, biologists and other researchers, there has been no change in the prognoses of primary malignant brain tumors like glioblastoma multiforme in the last 30 years. The five-year survival rate is less than 5% in glioblastoma despite combined surgery, radiotherapy and chemotherapy. After Ausman et al. stated that evolution of surgery and radiotherapy would have no significant value for the prognosis in malignant brain tumors in 1970, the trial of potential chemotherapeutic, genetic and immunologic therapy methods in appropriate experimental glioma models came into scene. These studies have been conducted with experimental glioma models worldwide and especially in the U.S.A. and Japan for the last 30 years (4,6,9).

In this study, we tried to understand these tumors better by forming a C6 rat glial tumor model and detecting their tumorigenicity, invasion and migration behavior.

MATERIAL and METHOD

C6 Rat Glioma Cells

The cells were provided by the American Type Culture Collection (ATCC – Maryland,USA). They were stored at 37 °C in a CO2 incubator in 5 cc flasks with 10% fetal calf serum (FCS)-added F12 medium (Sigma Chemistry, İstanbul) (8,17).

Preparation of the Passage

Before implantation, the medium-cell combination in flasks was poured into a beaker. 2 cc trypsin was added. Short-time friction was applied to free the cells from inside the beaker. The trypsin and cell mixture was then put into a centrifuge tube and 1,5 cc F12 medium and FCS were added. The mixture was centrifuged at 1000 cycles/minute for 3 minutes. 2 cc medium was added to the sediment and a suspension is made by mixing with a pipette. Cells were counted using a Tomo camera (8,9,14,17,19,20).

Rats

Eight Wistar and eight Sprague-Dawley male rats weighing 250-350 grams were used. The rats were provided by İstanbul University, Experimental Medicine Research Center (DETAM). Tumor cells were implanted to the rats in 10 µL suspensions containing 104 to 107 cells.

Implantation

Rats were anesthetized intraperitoneally using a ketamine and phentanyl combination. A 1 cm median vertical incision was made on the bregma. Skin and subcutaneous tissue were passed. The periosteum was elevated. A 4 mm burr hole was opened at the intersection of the points 1 mm anterior to the coronal suture and 3 mm lateral to the sagittal suture at the left side with 3 mm depth. Implantation was made to the left frontal area using a 22 G needle. The details of the technique is described elsewhere (3,10,11, 16, 21, 22, 24).

Cranial MR Imaging

Before decapitation, a cranial T2-weighted MR imaging was performed to all rats.

Decapitation and Fixation

Eight rats were decapitated 2 weeks after implantation and others third. All 16 brains were taken out as a whole with no damage (Figure 1,2) and sent to İstanbul University, Neuropathology Laboratory in 10% formalin. Paraffin blocks were prepared and 6 micron slices were made. Hematoxylin and Eosin were used to stain the slices. The slices were examined macroscopically and

microscopically. Glial fibrillary acidic protein (GFAP) was studied both in normal and tumoral tissue (9,19,20).

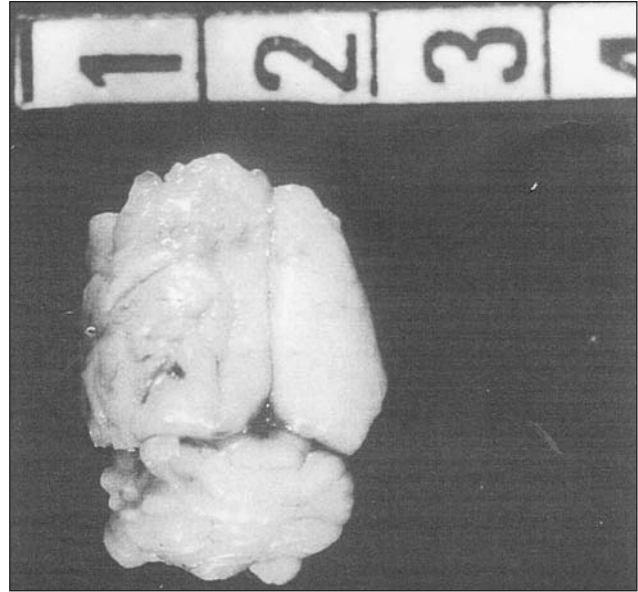


Figure 1: Macroscopic global view of a Wistar rat brain after decapitation

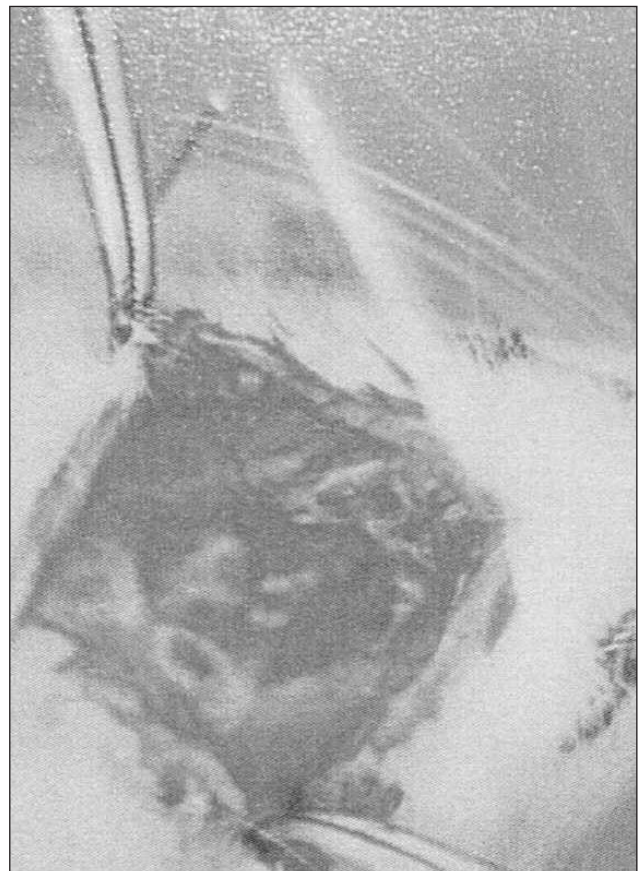


Figure 2: Decapitation process of the rat

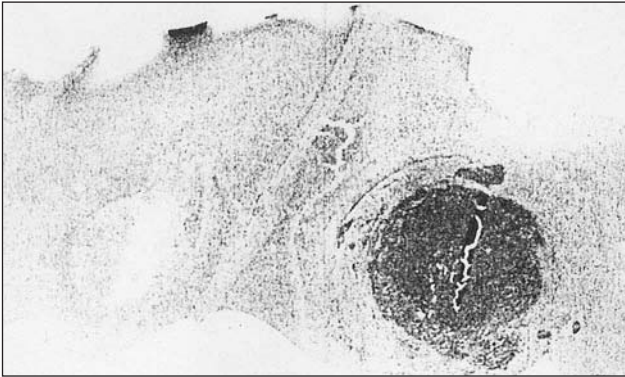


Figure 3: Macroscopic coronal section of the rat brain in Figure 1



Figure 6: x125 magnification of the microscopic view in Figure 5

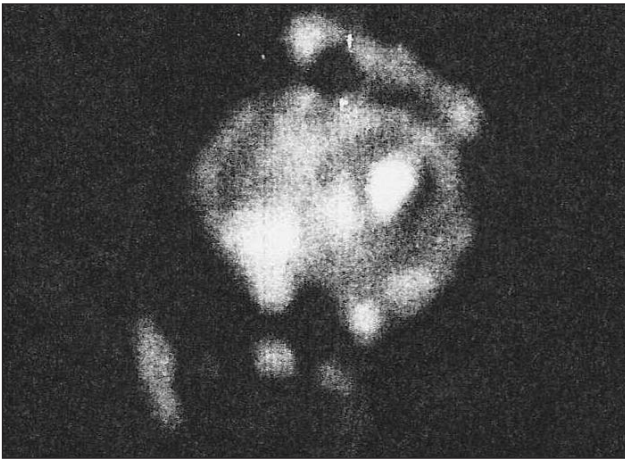


Figure 4: T2-weighted coronal cranial MRI

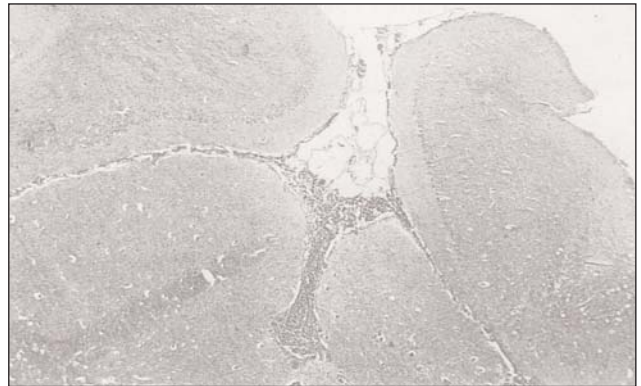


Figure 7: Microscopic view of a Sprague-Dawley rat brain after H&E stain (x32)

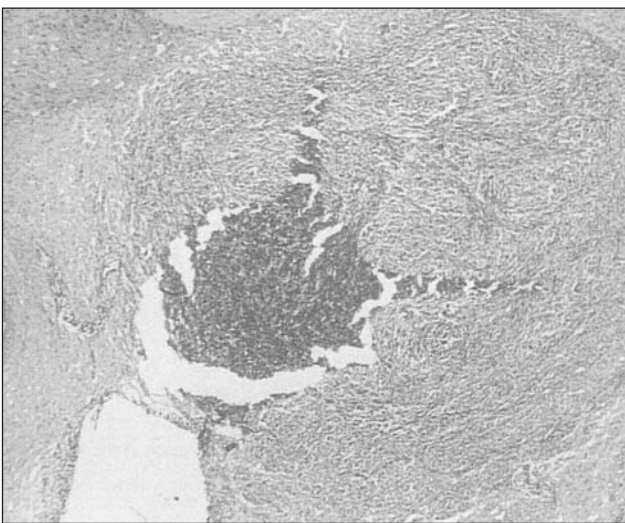


Figure 5: Microscopic view of a Wistar rat brain after H&E stain (x32)

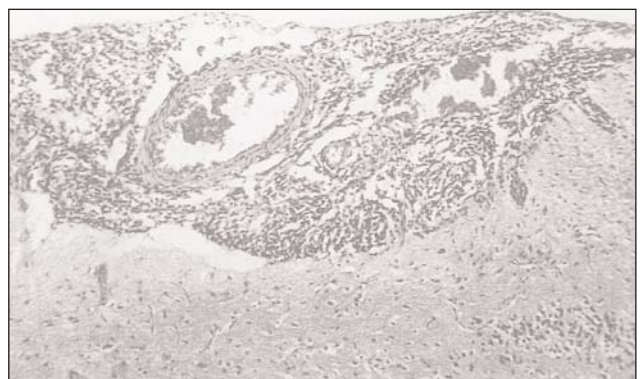


Figure 8: x125 magnification of the microscopic view in Figure 7

RESULTS

Tumor Formation

Tumor formation was evaluated by both macroscopic and microscopic histopathological examination. Tumor formation was observed in all rats. While the tumors had significant mass effect and more or less well-differentiated margins at the left frontal region (implantation site), there were tumor cell lines that did not form mass lesions but from place to place produced layers away from the implantation site at the periventricular zone, subarachnoid regions (Figure 7,8).

Invasion

106 and 107/10 μ L cell-implanted rats had differences in their brains visible with the naked eye. In the macroscopic global examination of the brain of a 106/10 μ L cell-implanted rat (Figure1), it was seen that the left hemisphere was larger and softer (edema) than the right hemisphere; the right hemisphere had smooth and slippery arachnoid whereas the left hemisphere had a rough and yellowish-gray arachnoid. This change covered the whole hemisphere except the left parasagittal superomedial region. This macroscopic finding was not observed in 104 and 105 /10 μ L cell-implanted rat brains. In the coronal sections of all rat brains tumor formation was observed in the left frontobasal regions with H&E staining. Microscopically, C6 rat glial cells showed the same staining characteristics as human glial tumor cells with H&E staining. Cells had round to oval with acidophilic nuclei but had partly bipolar, partly scattered cytoplasm. The tumor was quite well-differentiated from the surrounding tissue. Both in Sprague-Dawley and Wistar rats, there were layers of tumor cells away from the implantation site; at the brainstem, hippocampal region and ventricular system; especially the perivascular and perineural regions (Figure 7,8).

At the implantation site, all rats had tumors well-differentiated from the surrounding tissue, that showed intense arachnoidal spreading and the size of which was proportional to the number of tumor cells implanted (5,13,26,31)

Cranial MR Imaging

All rats underwent cranial MR examination before decapitation. T2-weighted coronal cranial MR image of a Wistar rat brain is seen in Figure 4. At the left frontobasal region, a tumor formation compatible with the macroscopic and microscopic

histopathologic examinations (Figure 3), with a hyperintense center and hypointense surrounding is visible and at the right temporobasal region, the turbid hyperintensity shows the migration region (Figure 4).

DISCUSSION

Histopathology

Although at first glioblastomas were thought to originate from primitive embryonal cells, it is now accepted that they consist of highly anaplastic mature glial cells and mostly astrocytes (23,32,33). Kernohan et al. use "astrocytoma grade 3 and 4" in their classification instead of the word "glioblastoma". Glioblastomas can secondarily form by an oligodendroglioma or astrocytoma gaining an anaplastic character or primarily when there is no differentiation sign. It is generally accepted that most are secondary tumors. The histopathological character of the cells in the experimental rat glial tumor model in this study shows significant resemblance with the human glioblastoma cells. C6 cell line shows astrocytic morphology (2,22,27,28) and it is widely used in neurobiology as it contains the glial tumor markers GFAP and S100 (1,4,8,9,10,11,24).

The tumors can be multifocal. In this circumstance there may be two or more tumor foci or a big tumor mass and small tumor foci like satellites. Microscopically, glioblastoma is richly cellular and highly anaplastic. Tumor cells are round, fusiform or polymorphic. Generally different cell types are observed side by side. Sometimes the cells are so anaplastic that it is impossible to tell whether the tumor is astrocytic but generally there are somewhat differentiated astrocytic regions. Infiltrative growth, necrosis, foci of hemorrhage and endothelial proliferation at the vascular walls are the predominant histological factors. The most characteristic finding of glioblastoma is the necrotic foci surrounded by tumor cells. Ventricular spreading of the tumor can be in the form of small nodules or a layer completely covering the ventricular ependyma. Metastatic tumor tissue in the subarachnoid region or in the ventricles can infiltrate the brain tissue or secondarily the spinal cord tissue. In this study the macroscopic and microscopic findings of glioblastoma were all histopathologically observed (Figure 1,3,5,6,7,8).

Invasion and Migration

Invasion and migration play an important role in

glioma pathogenesis. Fetal and differentiated astrocytes show invasion and migration (23,25). Laminin is a permanent element of the basal lamina and is active in invasion and migration (30). Laminin is a molecule needed for nervous system growth. At the same time it is the preferred substrate for astrocyte and neuronal growth (12). For migration, fetal and differentiated astrocytes should develop an intracellular organization (cytoskeleton) and provide motion. The most important element for motion is cellular extensions. Another mechanism needed for cell migration is the secretion of cell adhesion molecules. These molecules move the cell through the substrate. For the cells to invade a tissue and migrate, protease should be secreted (9,12). Plasminogen activators are types of proteases and are secreted by gliomas. The activators play role in migration and invasion. Tissue plasminogen activators are key enzymes in thrombolysis. They free the tumor cell from the matrix located around the tumor tissue containing blood clot and make invasion easier. Urokinase is a plasminogen activator involved in the activation of plasminogen to plasmin. This enzyme lyses other matrices between basal lamina and invasion site (12). Glial cells produce not only extracellular molecules active in invasion and migration like laminin, proteases and adhesion molecules, but also intracellular organizer molecules like S100 (17). This enzyme provides cell motility by starting intracellular calcium mobilization. Quantitative increase of this protein promotes migration.

Migration proteins of C6 glioma-astrocytoma cells and human malignant glioma cells are the same. Migration is through the basal lamina surface and parallel to the neural fibers. Human glioma specimens also show the same pattern on pathological examination (7,8,16,21,28,29). In this study migration was definitely shown. In all rats, whether Wistar or Sprague-Dawley, there was a layer of C6 cells away from the implantation site and they did not produce a tumor mass. The cells in layers were detected in the brainstem, hippocampal region and ventricular system; especially at the perivascular and perineural regions (Figure 7,8). This finding supports that the glioma cells have migration ability (7). The reason why surgery and radiotherapy are unsuccessful in glioblastoma can be the increased migration ability (11,22,32). Migration and the invasive character of the C6 glioma cells brings to mind the question whether

multiple tumors are of multiple origin or are the result of migration. This characteristic of the tumor also makes one think about what to do besides surgery. Maybe the answers to these questions will put an end to glial tumors.

Immunohistochemistry

Immunohistochemistry plays an important complementary role in glial tumor diagnosis (19,29). With the widespread use of the electron microscope, it was shown that some tumors could be histopathologically misdiagnosed. In situations like this, the importance of immunohistochemical methods using tumor markers for the diagnosis is well known (9) The most important tumor markers in glial tumors are: glial fibrillary acidic protein, vimentin, S100 protein and Leu 7.

In this study, GFAP positivity was evaluated in every rat group. Strong positivity is observed in the parenchymal tissue around the tumor, but the tumor cells gave a weak response. After the literature was reviewed, this weak positive response was thought to be because the astrocytes did not constantly produce the same amount of GFAP (11).

CONCLUSION

C6 rat glial tumor model shows behavior similar to glioblastoma multiforme. This feature has been shown histopathologically and radiologically in this study. Invasion and migration, which are the most important and prognostic characteristics of glial tumors were observed independently from the number of implanted cells. This finding has once again shown that other therapy methods should be developed in addition to surgery. This hypothesis is also supported by the fact that the prognoses of the glial tumors show no improvement despite surgery and radiotherapy. In conclusion, the experimental glioma model in our study has shown all the characteristics of the glial tumors written above and has thus been found appropriate to the standards in literature and for use in new studies.

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