EKSPERIMENTINIAI TYRIMAI

Histology of human glioblastoma transplanted on chicken chorioallantoic membrane

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***Key words**: glioblastoma; chicken embryo; chorioallantoic membrane; immunohistochemistry; inflammatory cells.*

***Summary.** Glioblastoma is the most malignant tumor in the range of cerebral astrocytic gliomas. A lot of experimental models are used to evaluate various properties of glioblastoma. Chicken chorioallantoic membrane model is one of them.*

Objective. To evaluate histology and survival of glioblastoma tumors taken immediately from operating theatre and transplanted on chicken chorioallantoic membrane.

Materials and methods. Glioblastoma samples obtained from 10 patients were transplanted onto 200 eggs. Overall, we used 15 tumors; only 5 of them were not glioblastomas as it was revealed later.

Results. The transplanted tumors survive up to 6 days. Transplants do not survive longer because during embryo's development the nourishing membrane dries. Transplanted glioblastomas exhibited the same features as original glioblastomas – necrosis, endothelium proliferation, cellular polymorphism – while transplanted glioblastomas also showed glial fibrillary acidic protein (GFAP), vimentin, Ki67, S100 protein, neurofilament immunoreactivity, and infiltration of macrophages (CD68) and T cells ($CD3^+$, $CD8^+$). Transplanted glioblastomas did not show any immunoreactivity of p53. Invasion of vessels from the chicken into transplanted tumor is not observed. Chicken erythrocytes did not appear within the transplants, and tumor cells invade chicken tissue at the minimum.

Conclusion. Our data show that transplanted pieces of glioblastoma survive with all cytological features. The presence of macrophages (marker CD68) and T cells (markers $CD3^+$ and $CD8^+$) can be registered in the transplant. The data revealed that transplanted glioblastoma remains as insulated unit, which survives from nourishment of the chorioallantoic membrane apparently only by diffusion. The features of original tumor-host reaction of the patient remained too.

Introduction

Glioblastoma is the commonest neuroectodermal tumor and the most malignant in the range of cerebral astrocytic gliomas (1, 2). The patient's course fully reflects the biological aggressiveness of the tumor. Thus in spite of multimodal treatment, progress in surgery, chemotherapy, and diagnostic abilities, the median postoperative overall survival does not exceed 12 months (1). Invasion and metastasis are hallmarks of most malignant tumors including glioblastoma, resulting from the interaction between tumor cells and the surrounding tissues. The activation of many genes and the expression of their products have been shown to be important in tumor progression. Advance in therapeutic strategy will derive from better insight in the specific pathogenesis of this neoplasm. The main features of glioblastoma are cell polymorphism and mitotic activity, vascular abnormalities, and remarkable necrotic foci. These tumors may also have extensive hemorrhages and infiltration of inflammatory

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cells. Existing in vivo glioblastoma models are based on the inoculation of glioma cells into rodent brain or the use of transgenic mice. However, these models suffer from variable growth rate and poor penetration, and are limited by the difficulty of obtaining morphological data. These models do not reflect properly the interaction between tumor and host that occurs in the human, accurate invasion processes, vasculature, gene expression profiling, and stoma interactions (3, 4). For decades, the avian model was the model of choice in experimental biology. The chick embryo chorioallantoic membrane (CAM) model is a well-established method for studying cancer, cell biology, immunology, and genetics (5–7). The CAM model is well-established method to keep explanted material alive and supply it with oxygen and nourishment. It is also used to grow various neoplasms, although glioblastoma material was used very rarely.

Glioblastoma has some typical immunohistochemical features: it shows immunoreactivity of intermediate filaments that are found in astrocytes such as glial fibrillary acidic protein (GFAP) and vimentin. The presence of both vimentin and GFAP in cultured glial and glioma cells has also been shown in many other studies. The coexpression of GFAP and vimentin has been proposed to be a general feature of astroglial cells both in vitro and in vivo, though it does not contribute to tumor development or progression (8–10).

Neurofilament proteins are major components of the neuronal cytoskeleton, ones of the main markers of neuronal tissue; however, their immunoreactivity is highly variable (11, 12). Tumor proliferation is shown by Ki67. In human astrocytomas, the Ki67 labeling index correlates well with other proliferation markers, histological grade, and prognosis. Protein S100 is a marker of neuroectodermal cells (glial cells also). It is common in other human tumors and is not specific to glioblastoma (13–16). Though p53 tumor suppressor gene is a very frequent target for genetic alterations in most human cancers, it is present in human gliomas only in about 30% of cases. However, this number suggests that it is involved in the development of gliomas (17–23).

The brain has specialized mechanisms of interaction between parenchyma and immune system, and glioblastoma itself creates an environment that is favorable for its growth by creating an immunosuppressive environment. Immune system cells – macrophages (marker CD68) and T cells (markers $CD3^+$ and $CD8^+$) – are present in glioblastoma. The percentage of macrophages infiltrating glioblastoma can reach up to 30% of tumor mass (24).

The aim was to investigate whether the glioblastoma tissue transplanted on chorioallantoic membrane survives with it main histological and immunological features.

Materials and methods

Tissue samples

Fresh samples of glioblastoma were obtained from the operated patients. These patients had both clinical and radiological diagnosis of glioblastoma. The fresh samples were carried to the laboratory in isotonic saline solution. They were transplanted on CAM within an hour after the samples were obtained.

Sample evaluation

In vivo transplants were photographed with a digital camera, through a stereomicroscope with cold light under magnification from 1.6 to 4 times depending on the sample. Samples of CAM together with tumor were cut at certain time intervals (every 24 hours) and fixed in 4% formalin solution. Fixed tissues were dehydrated in ethanol and xylol and embedded in paraffin. Tissue paraffin blocks were sectioned into 5-μm slices using a microtome. Half of the slices were stained with hematoxylin-eosin to visualize the tumor cells. Histological and immunohistochemical samples were evaluated through the light microscope under an original magnification of 4 to 40 times depending on the sample. The other slices were stained using immunohistochemistry – GFAP (glial fibrillary acidic protein, mouse anti-human 1:100, Dako), vimentin (1:400, Dako), neurofilament (1:100, Dako), Ki67 (1:100, mouse anti-human, Dako), S100 (1:1000, rabbit anti-human, Dako), p53 (1:100, mouse anti-human, Dako), CD68 (1:200, mouse anti-human, Dako), $CD3^+$ (1:300–1:500, rabbit anti-human Dako), $CD8^+$ (1:20, mouse anti-human, Dako) markers. All sections were deparaffinized according to protocol; endogenous peroxidase was blocked in 0.3% hydrogen peroxide in phosphate-buffered saline. After the procedure, all slices were placed in an automatic staining system (Optimax, BioGenex). All immunohistochemistry was done in the Institute of Neuropathology, RWTH Aachen University Clinics, using Optimax, BioGenex staining system.

Chorioallantoic membrane model

Glioblastoma tissue was transplanted onto the chorioallantoic membrane in the period of day 7 to day 9, when the chorioallantoic membrane is mature. Following transplantation, the window was covered

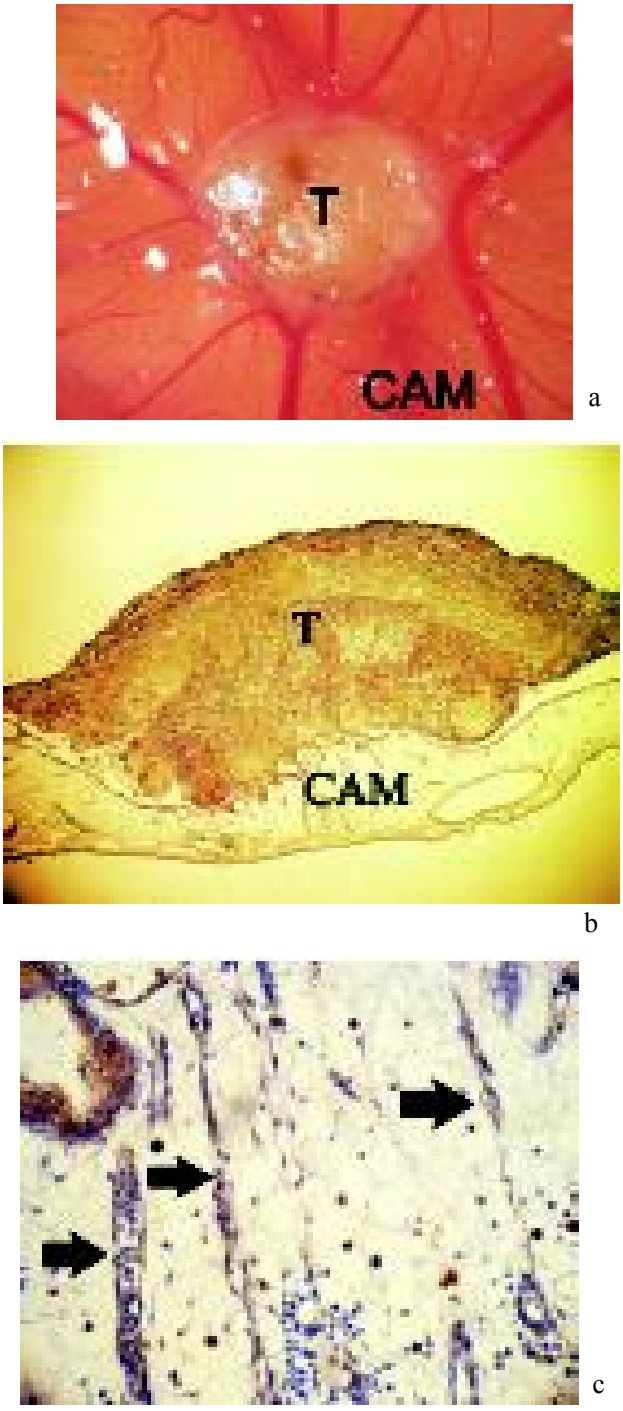


Fig. 1. **In microphotographs of low magnification, the view of transplanted glioblastoma on chorioallantoic membrane (CAM) is shown**

a – 72-h tumor (T) on CAM in vivo with vessels attracted to tumor and small vessels from CAM orientated in the direction of glioblastoma (original magnification, ×4); b – the histological overview of the transplanted tumor (T) nesting on CAM (original magnification, ×4); c – the tumor growth is supported by small vessels (arrows), which are attracted to tumor, but do not enter it (original magnification, ×25; both slices stained immunohistochemically for protein S100).

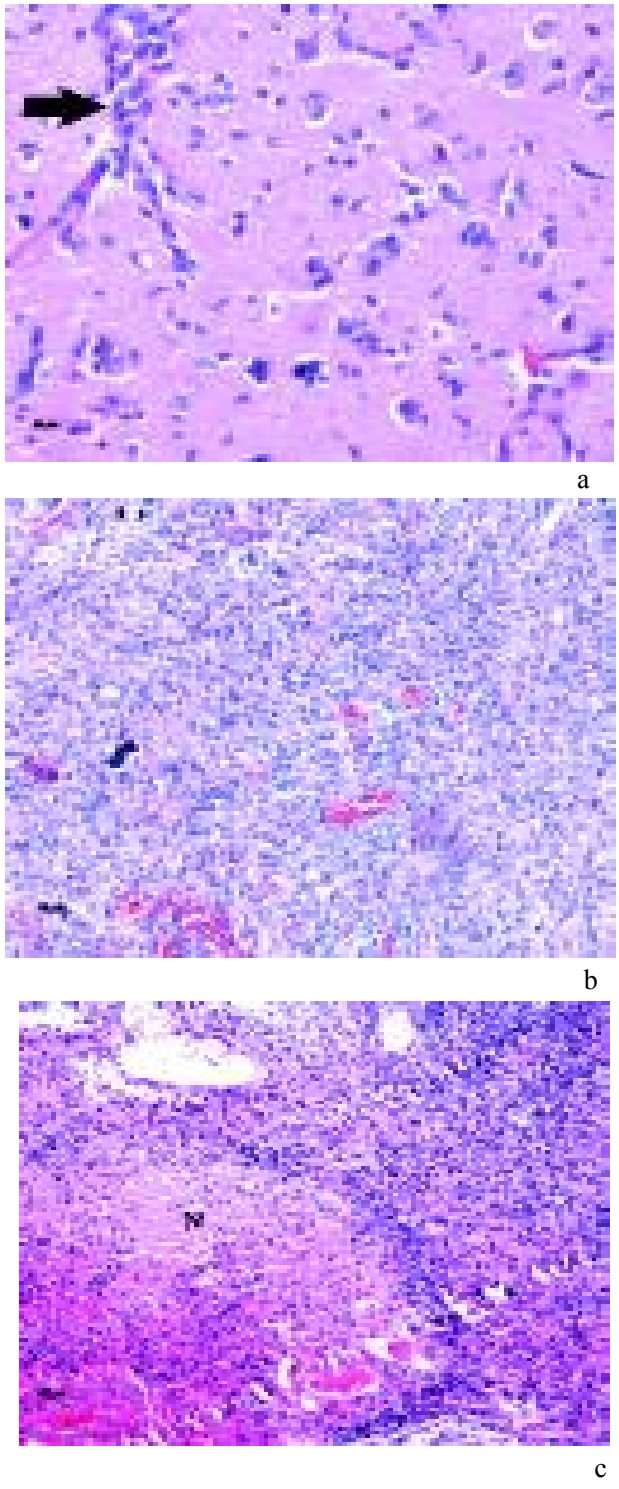


Fig. 2. **Microvascular proliferation (arrows) in glioblastoma transplanted on chicken chorioallantoic membrane (a) (original magnification, ×25)**

Cell variety (b) in original glioblastoma (original magnification, ×10) and typical view of glioblastoma cells in tumor transplanted on chicken chorioallantoic membrane together with the necrotic areas (N) (original magnification, ×10). Histological slices stained with hematoxylin-eosin.

again, and the egg was placed back in the incubator. The reactions of the transplant on the chorioallantoic membrane were registered by a stereomicroscope with cold light. At regular time intervals, the specimens were taken out, fixed, and stained. The transplants were taken out after 24, 48, 72, 96, 120, 144, and 168 hours following transplantation. All specimens were stained with hematoxylin-eosin, and then selected samples were stained immunohistochemically. The sample selection was done according to the tumor visualization – samples with the tumor cells and surrounding chorioallantoic membrane were selected. Immunohistochemical markers for evaluation were GFAP, vimentin, neurofilament, S100 protein, Ki67 and p53, CD68 macrophages, and $CD3^+$ and $CD8^+$ T lymphocytes; all markers were obtained from Dako (Denmark).

Results

In these experiments, we tried to show that the main features of original glioblastoma tumor taken from patients remain the same when the tissue is transplanted on the chicken chorioallantoic membrane.

In our study, glioblastoma samples taken from 10 patients were transplanted onto 200 eggs. During incubation period, 22 chicken embryos died (11%). Transplantation was successful in 158 eggs (79%). The other five tumors were not glioblastomas as it was later revealed by pathohistological evaluation, and they were not included into investigation. The transplants were taken out of eggs successfully at different time intervals, starting from 24 hours after transplantation. Glioblastomas transplanted on the chicken chorioallantoic membrane survived up to 6 days. Transplants do not survive longer because during embryo's development the nourishing membrane dries. Nucleated chicken erythrocytes did not appear within the transplants, showing that chicken vessels do not invade the transplant. Furthermore, tumor cells invade chicken tissue at the minimum.

Comparison of original tumor and transplanted tumor

Chorioallantoic membrane

The chorioallantoic membrane underlying the transplant appears as a sheet of loose connective tissue bordered on both sides by epithelial layers, containing vessels of different size. The transplant is at its bottom adhered to the CAM, but firm only at certain places, establishing a kind of hilum. Beneath the transplant, especially beneath the hilum, the CAM is broadened and vessels accumulate here. The longer the transplant survives on the CAM, the more and the larger are the vessels beneath the transplant as it can be directly observed using microscope. The vessels in the CAM contain nucleated erythrocytes and are, therefore, easy to distinguish from human erythrocytes. Within the transplant in the first days, the shadows of human erythrocyte are discernible. The hemoglobin soon disappears. Thick-walled vessels so prominent in the original glioblastoma in situ are found once more in the transplant but now in necrotic site.

Using the erythrocytes as criteria, no embryonic vessels enter the transplant nor do human vessels leave it. The increase in vascularization following transplantation is therefore merely embryonic reaction. Structures as well in the transplant as in the CAM are orientated radially to the hilum that points to the mechanical connection. At the hilum, an epithelial border between the specimens is present, although it could not be excluded that some gaps between the cells exist. Capillary may insert into the epithelial layer, but this appears only beneath the transplant. This phenomenon has never been observed in CAM regions at the distance to the transplant (Fig. 1).

Cells

Tumor cells are of higher density than normal brain tissue. Small and larger cells with atypical nuclear form and high nucleus-to-cytoplasm ratio characterize the picture. In addition, multinuclear giant cells are found. Because of this polymorphism, inflammatory cells are difficult to distinguish from tumor cells in hematoxylin-eosin-stained slices. To reveal this, immunohistologic staining is needed. Summarizing, glioblastoma demonstrates hypercellularity with cellular and nuclear pleomorphism. Transplanted tumors also showed microvascular proliferation, cellular variety, nuclear atypia, and even mitotic cells (not shown) very similar to original glioblastoma (Fig. 2).

All transplanted glioblastomas expressed astrocytic intermediate filaments – were positive for vimentin and GFAP though in different degrees approving their glial origin (Fig. 3). In some cases, expression of these filaments was diminishing with tumor growth on chicken CAM, probably due to increasing necrotic zones caused by nutrition problems.

We found Ki67 only in a few transplants assuming that with increasing time of growth on CAM, the necrotic areas of transplant were becoming wider, and thus the proliferation of the cells was limited. Protein S100 was found in majority of transplanted glioblastomas. Neurofilament protein was also positive indicating the neuronal tissue in transplanted glioblastomas (Fig. 4).

Transplanted glioblastomas did not demonstrate the expression of p53 protein, but the detection of this protein in original glioblastomas was not performed either.

Endothelial proliferation

Vessel proliferation is a typical feature of glioblastoma. It is characterized by multilayered, mitotic active endothelial cells, smooth muscle cells, and pericytes. This proliferation is believed to influence the behavior of tumors and their aggressive growth phase (Fig. 5). In transplanted glioblastomas, vessel proliferation was found, but the process was not very typical because the vessels were not proliferating, but getting necrotized because of transplant nutrition problems (not shown).

Necrosis

Multiple areas of necrosis are found in glioblastoma. Usually two types of necrosis are encountered. The first ones are elongated, serpentine necrotic foci surrounded by radially orientated, densely packed, small fusiform glioma cells in pseudopalisading pattern (Fig. 6). These are histological hallmark of glioblastoma. The other ones are large and extensive area of necrosis containing necrotic tumor cells and vessels; these are because of insufficient blood supply and poor nutrition.

Immune system cells

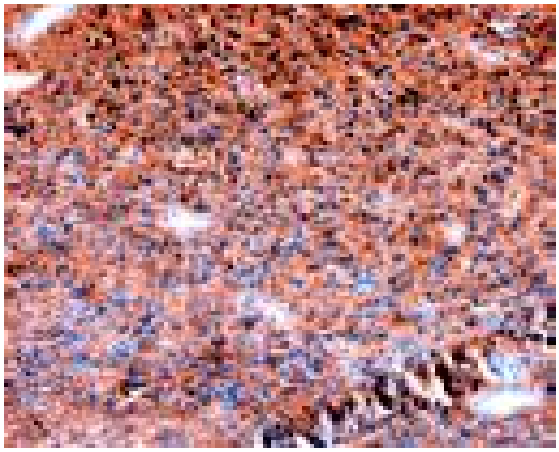
Macrophage and T cell infiltration has been reported in human glioblastomas. Glioblastomas transplanted on chicken CAM also demonstrated the infiltration of macrophages (CD68). The presence of $CD3^+$ and $CD8^+$ T lymphocytes was also confirmed (Fig. 7).

Discussion

Glioblastoma is a tumor grown on chicken CAM very rarely; in contrast to this, other human neoplasms are transplanted more often. Chick embryo assays are used to study glioblastoma, but up till now, only glioblastoma cell lines (4) and minced tissue (23) were studied on the chorioallantoic membrane. In some cases, glioblastoma cell lines are injected into chicken's brain ventricle (24). The natural immune-deficient environment of the developing bird (before day 18) is in favor of these xenograft experiments. Usually cell lines are used, which are easy accessible under laboratory conditions, but the correspondence to the original glioblastoma is not self-evident. To avoid these uncertainties, we took glioblastoma specimens immediately from the operation theatre and placed them onto CAM of hen's embryo for keeping the original tumor tissue alive. Our data of previous studies showed that glioblastoma tissue taken straight from the operating room survived on chorioallantoic membrane (25).

At first the question arises whether the cytological and histological features of the glioblastoma mentioned above following transplantation remain the same as those of original glioblastoma during overall embryo's development. The glioblastoma is of course a xenograft, but the embryo during transplantation is still before developing its own immune system (5, 26, 27). The promising perspective of this new approach is that success in therapeutic relation with the explanted tissue may be without too high risks transferred to the patient, from which the specimen was taken.

In these experiments, chicken vessels do not invade transplanted tumors as distinct from cell line experiments suggesting that glioblastoma survives but does not proceed growing. The glioblastoma transplanted on CAM remained histologically isolated, and it means it does not invade the host nor does the host invade the graft. In most of the cases, only epithelium proliferation of host was seen with only a few tumor cells invading the membrane. The only action of the host seems to be nourishment and oxygen supply to transplant. In accordance, the host is by no means harmed by transplant. The development of embryos remained normal. The tumor cells within the transplant keep their original cytological characteristics – cell variety, nuclear pleomorphism, and mitosis – and even the histological characteristics of glioblastoma were sustained. Surprisingly, even cells of the immune system, macrophages and T cells, persist up to 6 days in the transplant. These cells do not derive from the host because chicken T cells do not react with our applied agents. Regarding the histopathological features of glioblastoma, vascularization within the transplant is abolished, and invasion of healthy tissue cannot be determined because the area of necrosis increases due to experimental conditions. The area of CAM, where the transplant is settled on, is enlarged and its vascularization is continuously increased during the days, and this is a common response of chorioallantoic membrane to transplants. By this manner, nontumor transplants are able to grow on CAM. In contrast, our glioblastoma transplants become more and more necrotic during the days. The nourishment of the glioblastoma piece on the CAM is only achieved by diffusion, because no circulation in the trans-



a

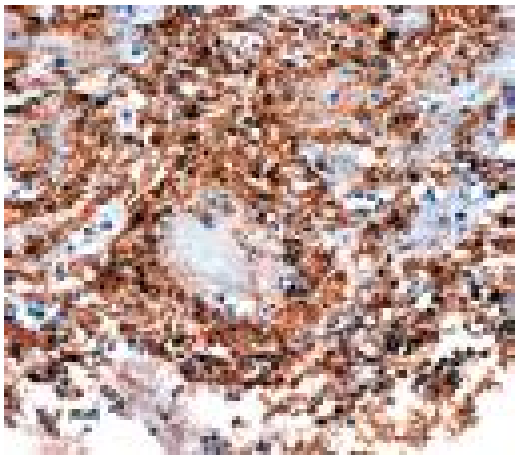


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Fig. 3. **Glial fibrillary acidic protein (a) and vimentin (b) expression in glioblastoma transplanted on chicken chorioallantoic membrane (original magnification, ×10; slices stained for glial fibrillary acidic protein and vimentin, respectively)**

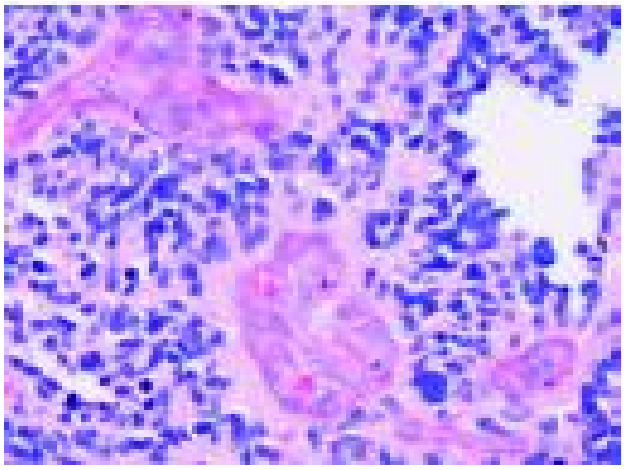
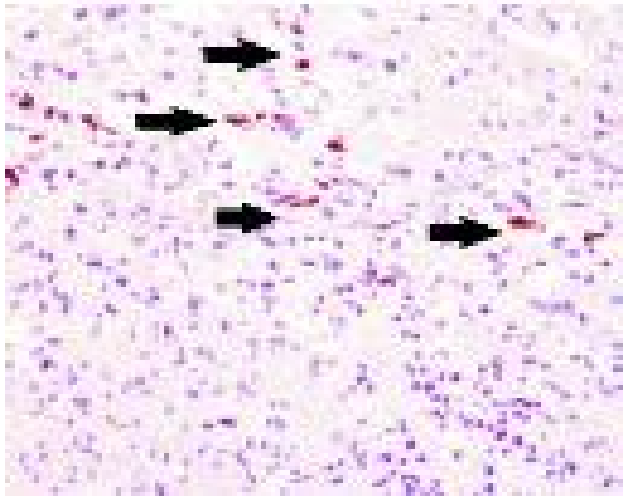


Fig. 5. **Endothelium proliferation in original glioblastoma**

Original magnification, ×25;
histological slide stained with hematoxylin-eosin.



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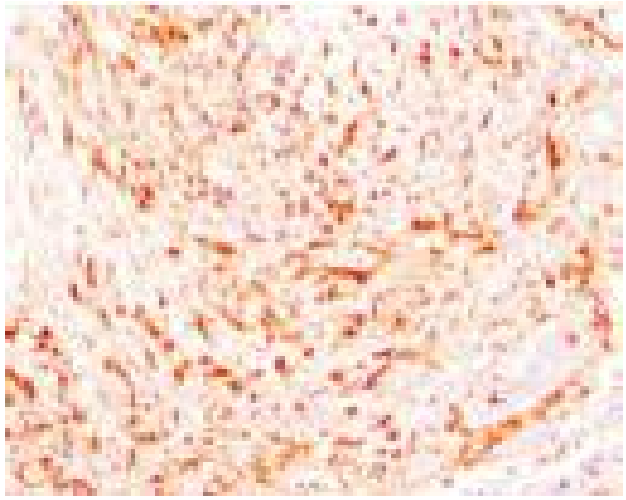

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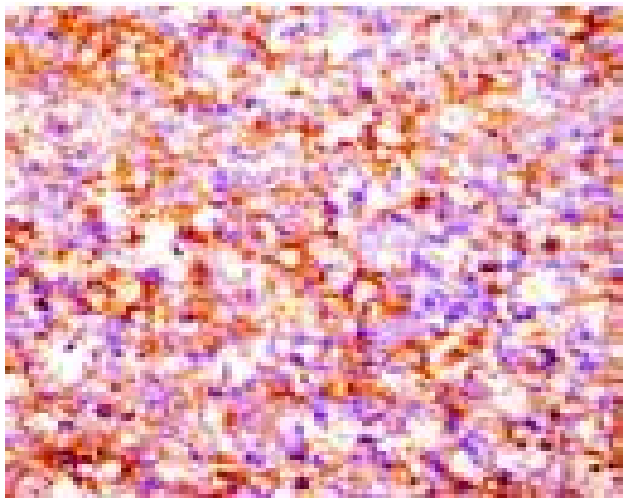

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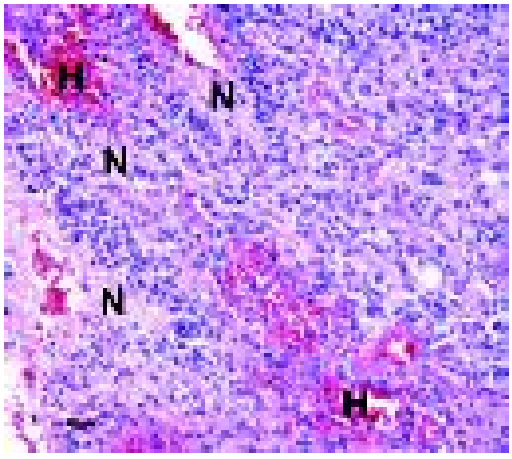
Fig. 4. **Immunohistochemical demonstration of Ki67 (arrows) (a), S100 (b) proteins, and neurofilament (c) (original magnification, ×10; slices stained for immunohistochemical markers)**



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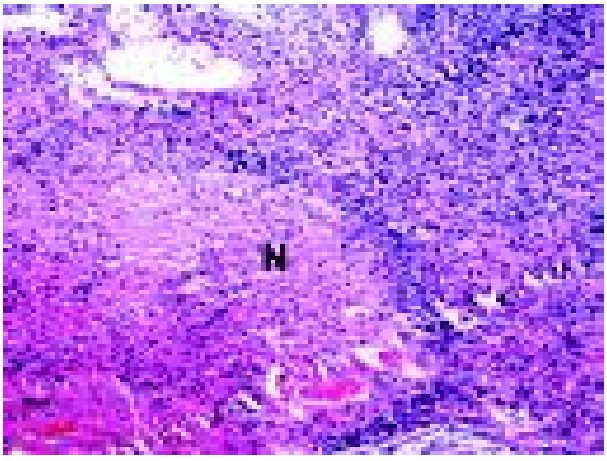
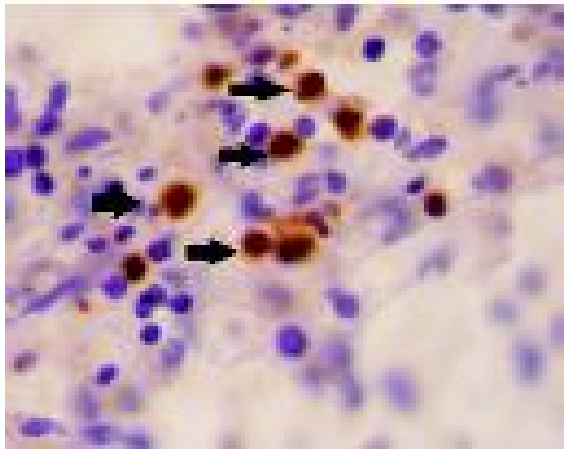


b

Fig. 6. **Micro hemorrhages (H) and typical necrosis (N) with pseudopalisading phenomenon in both human glioblastoma (a) and glioblastoma transplanted on chicken chorioallantoic membrane (b) (original magnification, ×10; histological slides stained with hematoxylin-eosin)**

plant is maintained. Increasing necrosis because of hypoxia, accompanying better blood supply, is a contradiction. The mechanism of necrosis in the transplant, therefore, cannot be easily explained. Furthermore, the role of the macrophages and the T cells in the transplant is unclear, which are still vital all the time. Especially the macrophages are present in large number. The macrophages are extremely loaded by ingested material. Regarding the immune system, it seems that some kind of tumor-host reactions of the patient still remained.



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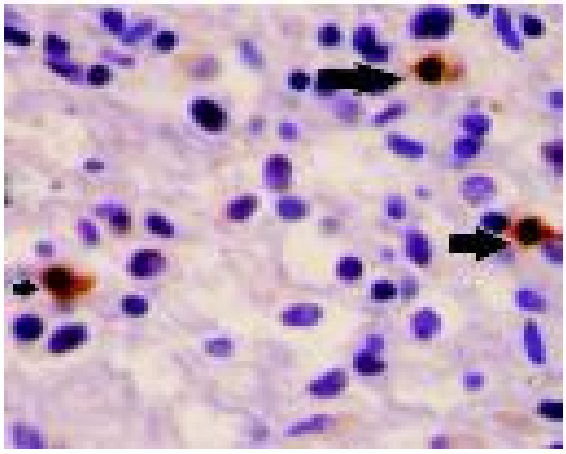

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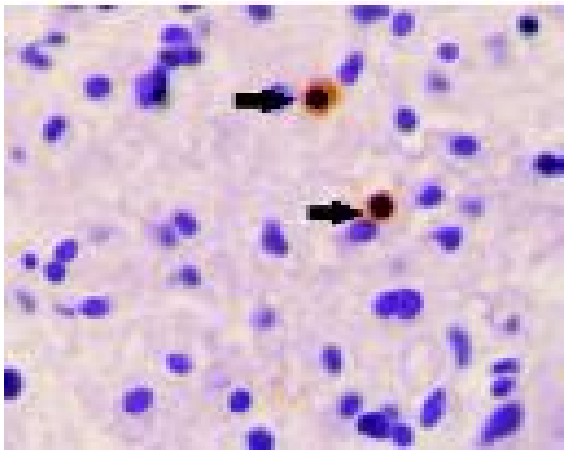

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Fig. 7. **Infiltration of inflammatory cells (arrows) in glioblastoma transplanted on chicken chorioallantoic membrane: macrophages CD68 (a), $CD3^+$ T lymphocytes (b), and $CD8^+$ T cells (c) (original magnification, ×40; slides stained with immunohistochemical markers)**

Conclusion

Our data show that transplanted pieces of glioblastoma survive with all cytological features. The presence of macrophages (marker CD68) and T cells (markers $CD3^+$ and $CD8^+$) can be registered in the transplant. The data revealed that transplanted glioblastoma remains an insulated unit, which survives from nourishment of the chorioallantoic membrane apparently only by diffusion. The features of original tumor-host reaction of the patient remained too.

Žmogaus glioblastomos, transplantuotos ant viščiuko chorioalantojinės membranos, histologinės savybės

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Raktažodžiai: glioblastoma, viščiuko embrionas, chorioalantojinė membrana, imunohistochemija, uždegiminės ląstelės.

Santrauka. Glioblastoma yra dažniausia smegenų naviko forma ir kartu pats agresyviausias iš visų smegenų navikų, sąlygojantis didžiausią pacientų neįgalumą ir mirtingumą. Glioblastomai tyrinėti naudojami įvairūs eksperimentiniai modeliai. Viščiuko chorioalantojinės membranos modelis yra vienas iš jų. Šis modelis nuo seno žinomas eksperimentinėje biologijoje.

Tyrimo tikslas. Įvertinti glioblastomos audinio, persodinto ant chorioalantojinės membranos, histologines savybes ir išgyvenimą.

Tyrimo medžiaga ir metodai. 10 operuotų pacientų glioblastomos audinio pavyzdžiai persodinti ant 200 embrionų chorioalantojinės membranos. Iš viso persodinta 15 navikų, tačiau penki iš jų buvo ne glioblastomos, kaip paaiškėjo ištyrus biopsinę medžiagą.

Rezultatai. Persodinti navikai ant chorioalantojinės membranos išgyvena iki šešių parų. Vėliau dėl embriono raidos ypatybių membrana išdžiūva ir navikai netenka mitybos. Persodinti navikai turėjo tas pačias morfologines savybes kaip ir tikrosios glioblastomos (ląstelių polimorfiškumą, nekrozę, endotelio proliferaciją). Persodintos glioblastomos buvo tiriamos ir imunohistochemiškai: glijos skaidulinio rūgštinio baltymo (GFAP), vimentino, Ki67, neurofilamento, S100 baltymo, makrofagų (CD68) ir limfocitų žymenimis ($CD3^+$, $CD8^+$). P53 žymeniui visi persodinti navikai buvo neigiami. Chorioalantojinės membranos kraujagyslės neįsiskverbia į naviką. Viščiuko eritrocitų navikuose nerasta. Pažymėtina, jog naviko ląstelės minimaliai įauga į chorioalantojinę membraną.

Išvados. Persodintos glioblastomos išlaiko citologines tikrųjų glioblastomų savybes. Transplante randama makrofagų ir limfocitų. Duomenys rodo, jog persodinta glioblastoma išlieka izoliuotu vienetu, kuris išgyvena dėl mitybos per membraną difuzijos būdu. Kai kurios naviko „šeimininko“ reakcijos išlieka ir chorioalantojinėje membranoje.

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