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MORPHOLOGY OF HUMAN GLIOBLASTOMA MODEL CULTURED *IN OVO*

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Abstract

The aim of this study was the morphological characterisation of glioblastoma multiforme tumour grown *in ovo*. Tumour cells (U-87 MG) were implanted on the chorioallantoic membrane of chicken egg. The structural features of cultured tumours resembled the spontaneous glioblastoma multiforme; however, the differences were also indicated. Our results confirm applicability of *in ovo* culture in tumour genesis studies. The described novel model may be profoundly helpful for the future research on molecular mechanisms of tumour growth inhibition.

Key word: glioblastoma multiforme, U-87 MG, *in ovo* model, choriaoallantoic membrane.

Glioblastoma multiforme (GBM) is the most commonly recognised primary tumour of the human central nervous system. Due to its infiltrating development, total resection of this tumour has not been reported and life expectancy after its diagnosis is about one year. Malignant characteristics (WHO IV grade) and localisation of its growth make it an important scientific challenge to discover new drugs inhibiting the development of GBM (12). The research of GBM biology and its inhibition is commonly performed *in vitro* (13). Due to limited response of cultured tumour cells, a high demand for animal model studies is recognised. Most of the investigations using animal models are conducted on rodents (5). We propose an innovative model of the chicken embryo *in ovo*. Recent research performed *in ovo* is not only conducted in experimental embryology and toxicology (16, 23) but also in advanced oncology studies (23). The advantage of *in ovo* experiments in oncology research is the low percentage of transplant rejection due to late development of bird embryo's immune system (22, 25). The presented *in ovo* model has been established for the studies of antitumour drugs (21) and angiogenesis mechanisms in number of tumour types (17). However, the glioblastoma multiforme as the novel research model has not been characterised yet. The aim of the study was to evaluate the morphology of glioblastoma multiforme cultured *in ovo*.

Material and Methods

Cell culture. Human glioblastoma multiforme cell line U-87 MG (ATCC, N^{o} HTB-14) was incubated under standard conditions (37^{0}C, 5% CO_2), in Dulbecco's Modified Eagle's Medium (Sigma Adrich) with the addition of foetal bovine serum (Sigma Aldrich) and antibiotics. Before transplantation to chicken embryos, the cell culture was treated with tripsin and EDTA. Then, cells were centrifuged (1,200 rpm/5 min) and dispersed in culture medium in concentration of 5 million/20 µL.

Chicken embryo culture. The experiments were performed on fertilised chicken eggs of *Gallus gallus f. domestica* meat race (Ross 308). The eggs were incubated in the incubator ALMD-1N3-7 with automatic egg rotation system (one full rotation per hour) at 37^{0}C and 70% humidity.

Implantation of GBM cells on the chorioallantoic membrane (CAM) of the chicken eggs. The process of implantation of GBM was performed during 6th d of the eggs' incubation in laminar hood under sterile conditions. 0.5 cm^2 hole was cut in the eggs shelf after cleaning it with potassium permanganate. Then the inside parchment membrane of the air chamber was dissected.

Silicone ring with estimated number of 5 million tumour cells dispersed in 20 µl drop of culture media was placed in the area of formed blood vessels.

The prepared eggs were protected by permeable plaster (POLOPOR, 3M Vicoplast) and moved in the incubator (37^0C and 70% humidity). The control of the tumour growth was conducted 6 d after the implantation (Fig. 1).

Tumou isolation. After the 18^{th} d of incubation, the chicken were terminated and the tumours (Fig. 2) were fixed in 4% buffered formalin.

Histological slide preparation. After the fixation, samples were embedded in paraffin, then cut into 4 µm sections, and stained with haematoxylin and eosin.

Histopathological examination. The histopathological examination, based on WHO criteria, was performed under the Nikon Eclipse 80i microscope. Mitotic index was assessed as number of mitotic figures in 10 visual fields (40x). In case of each slide, the index was counted 3 times and average number was estimated.

Results

The shape of the grown tumours was mainly oval and their structure was lobular. The consistency of the tumours was found hard but elastic. Tumour surface was smooth.

A few cases of tumours formed outside the silicone ring were found. A tendency of the tumour cells to diffuse into the egg white and settle away of the ring was observed. Nevertheless, the tumours were always located nearby the blood vessels (Fig. 5). The cases of tumours formed in different egg areas, not always in the adjoining location, and tumours located in association with the yolk sac were found. All tumours presented the tendency of fast growth due to their location in blunt ends of eggs.

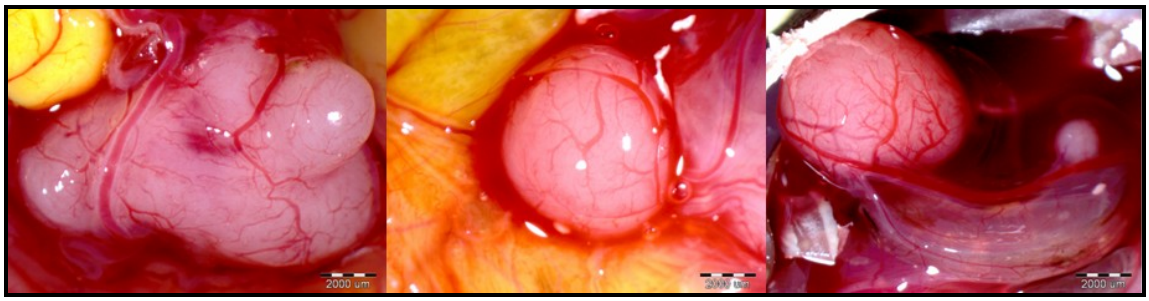


Fig. 1. Glioblastoma multiforme grown on the chorioallantoic membrane.



Fig. 2. Isolated tumours.

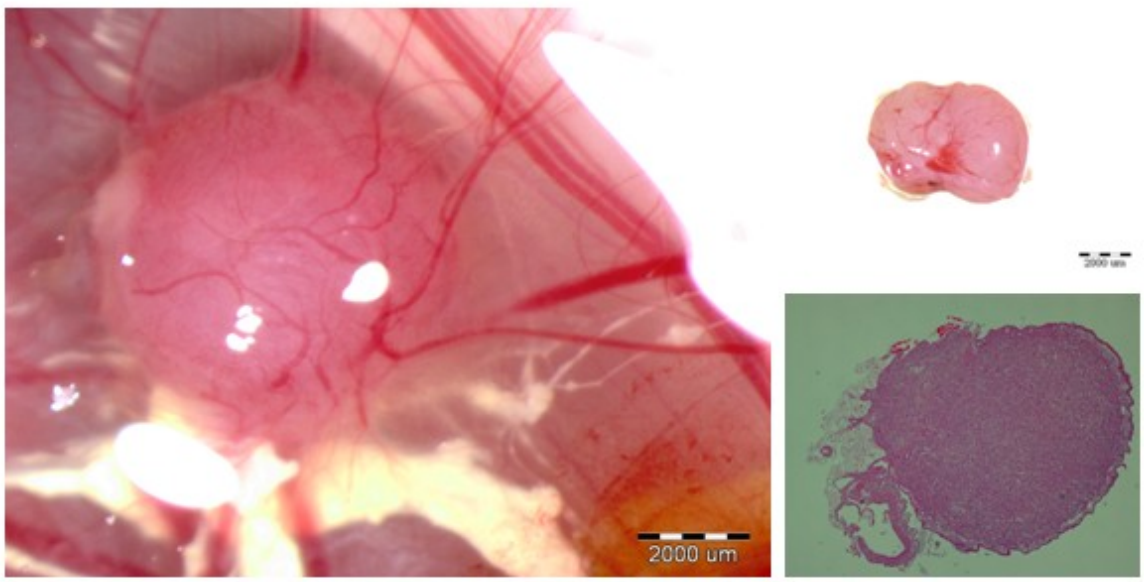


Fig. 3. Glioblastoma multiforme structure.



Fig. 4. Isolated tumours – diversity in the shape and size.

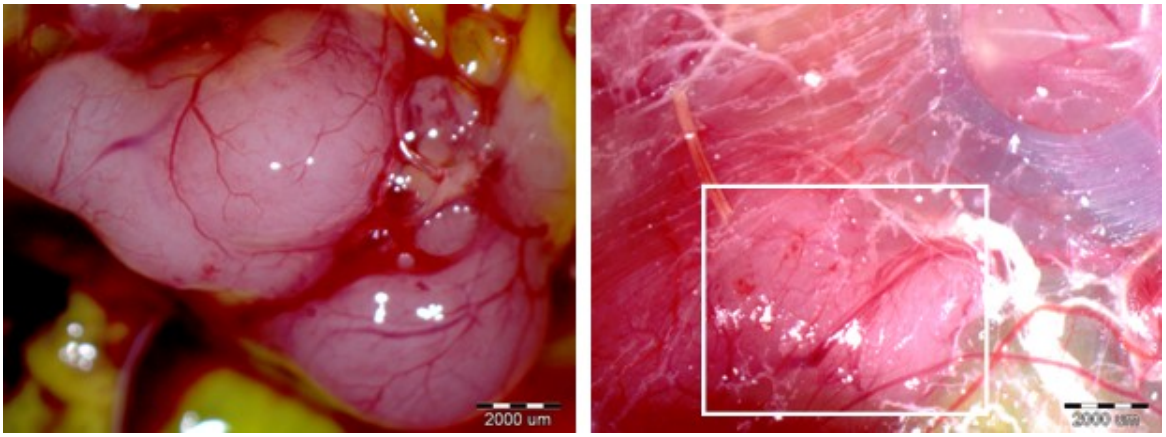


Fig. 5. Tumours formed in different areas of egg.

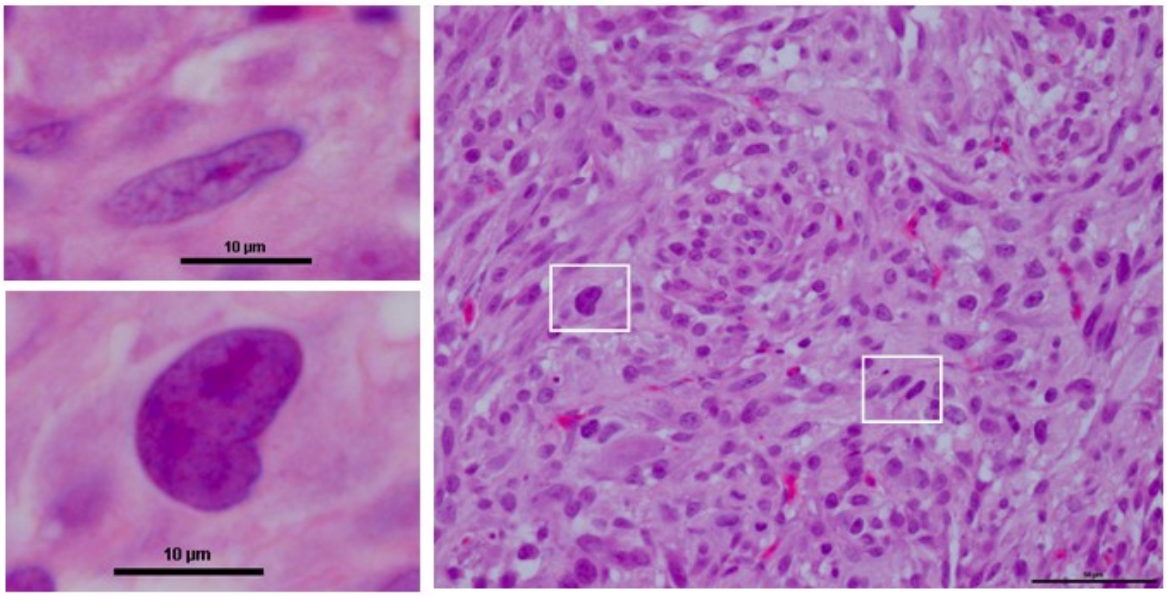


Fig. 6. GBM polymorphism with indicated giant cells.

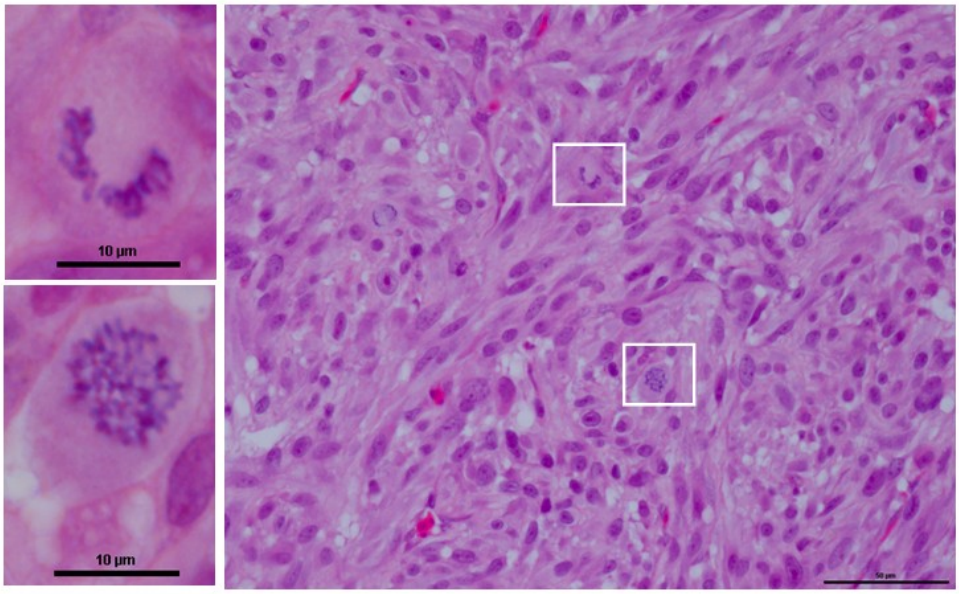


Fig. 7. GBM mitotic figures.

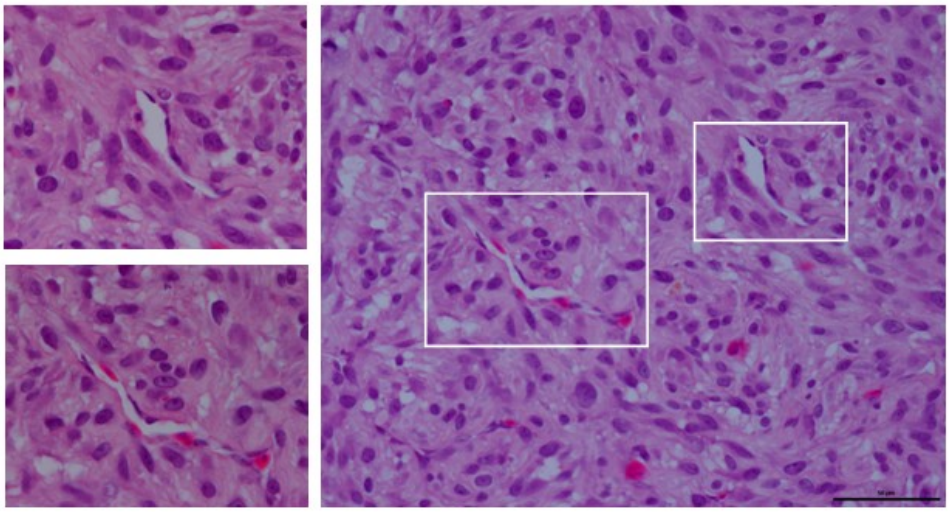


Fig. 8. Blood vessels formed in tumour.

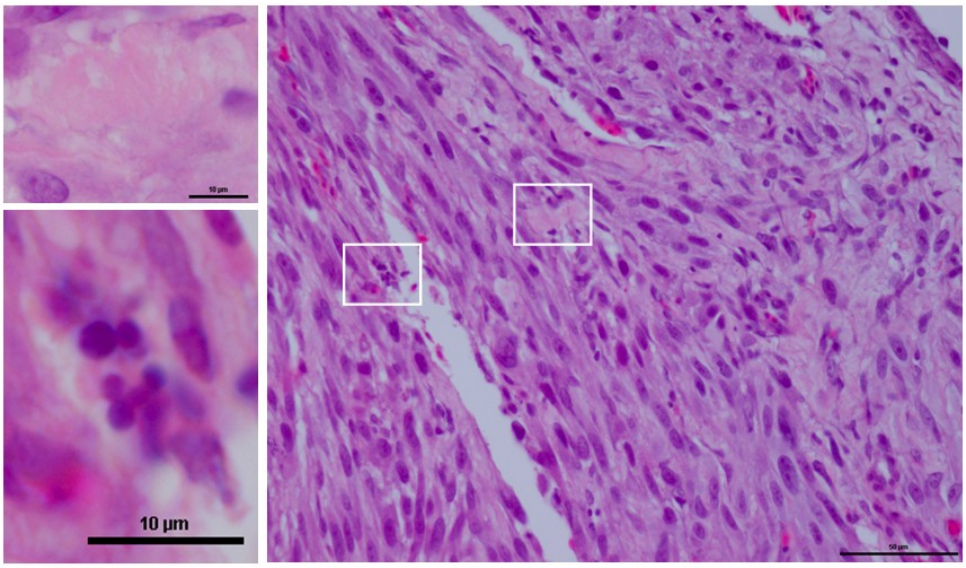


Fig. 9. Necrotic cells (upper picture on the left-hand side) and apoptotic bodies (lower picture on the left-hand side).

U-87 MG cells revealed high polymorphism of size and shape (Fig. 6). Nuclei demonstrated different characteristics of atypia and most of them possessed large and centrally positioned nucleoli in the number of 3 to 5 and rarely 1 or 2. The histological analysis revealed presence of single giant cells as well as acidophiles and lymphocytes infiltration.

The grown tumours revealed high mitotic activity. The mitotic index in the examined tumours varied from 3.7 to 5. Both physiological and pathological mitotic figures were observed (Fig. 7).

In all examined cases, the areas with elevated angiogenesis on tumour surface and its parenchyma were observed (Fig. 8). However, newly formed capillaries did not reveal glomerulus shape characteristic for the spontaneous tumour. No tumour cells were noted in newly created blood vessels.

In some cells of the grown tumours apoptotic bodies were found (Fig. 9). Small necrotic areas were also observed; however, no pseudopalisading sites, characteristic for the human glioblastoma were found (Fig. 9).

Discussion

The first reports of mammalian tumour cells's transplantation to chicken chorioallantoic membrane (CAM) were published in 1912 (14). However, the acceleration of the *in ovo* model research for oncogenesis was observed at the beginning of the recent century (9). Until 2010, only a limited number of studies have described tumour types predisposed for *in ovo* growth. It was shown that the cells intraoperatively isolated from tumours, such as: anaplastic astrocytoma, glioblastoma multiforme, oligodendroglioma, meningioma, ependymoma, lymphoma, and medulloblastoma, can be successively grown on embryonic CAM. It was observed that the most intense growth *in ovo* takes place after the implantation of benign neoplasm with no neuroectodermal origin (meningioma). The dynamics of the malignant neoplasm growth *in ovo* is significantly lower comparing to the benign neoplasm (18). The study of Tereseviciūte *et al.* (20) showed that glioblastoma multiforme explants grown *in ovo* develop large capillary net and may reveal necrotic changes. The work confirmed that GBM grown *in ovo* closely resemble the morphology of the tumour intraoperatively collected from humans (20). Balčiūnienė *et al.* (1) were able to transplant the tumour collected during the resection process. Tissue implantation was performed on 7 to 9 d of the chicken embryo development. Tumour was isolated after 24, 48, 72, 96, 120, 144, and 168 h after implantation. Due to the process of chorioallantoic membrane drying, the transplanted tumours survived no longer then 6 d. Tumours cultured under such conditions did not reveal the blood vessels net in developed bird embryo. The cell density was significantly higher than in the physiological human brain nerve tissue. Moreover, the histological analysis disclosed the presence of the multinucleated giant cells. Immunohistochemical study of Balčiūnienė *et al.* (1) presented the abundance of immunological cells (CD68 macrophages, $CD3^+$ and $CD8^+$ lymphocytes) in this tumour.

Comparative histology of the tumours grown *in ovo* was also presented (2). CAM was implanted with human osteosarcoma cells lines, such as: HOS, MG63, MNNG-HOS, OST, SAOS, SJSA1, U2OS, or ZK58. The tumours sized over 2 mm with evident vascularisation area on their surface were considered as positive results. The highest tumour growth with low mortality rate at the same time was achieved by implanting the cells of the MNNG-HOS line on CAM. All formed tumours possessed the ability to penetrate the membrane, as well as they were characterised by invasive growth and strong angiogenic response. One of the key factors for the experiment efficiency was the amount of cells passaged into the egg. According to the presented results, higher concentration of the cells decreases the bird embryo survivability. The highest efficiency, considered as the number of formed tumours, was achieved when the tumour cells were implanted at a concentration of 4.0×10^6/0.1 ml and 8.0×10^6/0.1 ml per egg. The success of the GBM culture *in ovo* was also conditioned by the time of the cell passage on CAM. The highest survivability factor (91.67% and 100% accordingly) was observed when the implantation was performed on 9^{th} and 11^{th} d of embryos' development. The process performed till the 5^{th} d of embryo growth correlated with low survivability and decreased tumour growth (24). In other reports on glioblastoma multiforme (cell line U-87 MG), implantation into the egg was conducted on 10^{th} d of the chicken embryo growth (11, 14) and examined on day 17 (11).

It was also presented that chicken nestling were hatched out with previously implanted tumour cells. However, the experimental animals were not able to survive longer than 3 weeks, probably due to the tumour growth with infiltrating characteristics (6). The advantage of U-87 MG spheroids in *in ovo* experiments was also described. The efficacy of the implantation depended on the size of the microtumours used in the research. It was found, that the best results, expressed by tumour high angiogenic potential, were achieved by implantation of 500 μm to 1 mm spheroids (7). The established methods of direct glioblastoma cell administration to embryo cerebral ventricle or nude mouse forebrain were also presented (3, 26).

In the presented study, histological characteristics of the glioblastoma multiforme grown *in ovo* showed several typical features of the human tumour (10). However, we noticed differences in localisation of pseudopalisading sites and the necrosis range. The absence of the extensive necrotic areas was most probably the result of the simple diffusion of elements from chorioallantoic membrane. According to the literature, tumours formed after implantation of U-87 MG cells into nude mouse brains do not possess the necrotic areas. Among the commercially available cell lines (U251, GL26, U-87 MG or CNS-1), only U-87 MG cells do not present the ability to induce the necrosis (3); however, Strojnik *et al.* (19) showed that only 20% of tumours cultured *in ovo* presented the necrotic areas and presence of lymphatic cells.

Xenotranplantation of cell line U87 to the forebrain of nude mice was also described by Zarnescu *et al.* (26). The grown tumours possessed the polygonal and elongated glial cells with oval and round nuclei and numerous nucleoli. Similarly to the result of our study, the proliferating cells and pathological mitotic figures were present. The presence of the apoptotic cells and bodies was also observed (26).

The absence of correlation between p53 expression and Ki-67 index of the U 87 cells *in ovo* was presented by Porter *et al*. (15). There are also conflicting reports describing *in vivo* GBM mitotic activity (8, 15). In our study, mitotic index ranged between 3.7 and 5 and was found higher than the activity of the baboon GBM (mitotic index - 1, 1-2, 2-3 mitotic figures per field of view, accordingly) (15). However, the mitotic activity presented in our study was lower than that observed in human GBM (in six cases with average survivability over 5 years) where the mitotic index was estimated as 8-16 figures in 10 visual fields (8).

The results of the presented research confirm the applicability of glioblastoma multiforme *in ovo* culture. The described model may be profoundly useful for the studies on the molecular mechanisms of the tumour growth inhibition.

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