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Proliferative and Apoptotic Activity of Glioblastoma Multiforme Cells Cultured on *In Ovo* Model

KAJA URBAŃSKA, JUSTYNA SOKOŁOWSKA, MACIEJ SZMIDT and PAWEŁ SYSA

Division of Histology and Embryology, Department of Morphological Sciences, Faculty of Veterinary Medicine, Warsaw University of Life Sciences, Warsaw, Poland

Abstract. *Aim: The influence of antitumor compounds on glioblastoma cell activity can be successfully investigated on an in ovo model. To consider an in ovo model as a reliable tool for estimation of anticancer drug efficacy, the biological activity of tumors growing in such conditions should be comparable to spontaneous cases. The aim of the present study was to evaluate the biological characteristics of glioblastoma multiforme (GBM) tumors – defined as their proliferative and apoptotic activities – growing on an in ovo model. Materials and Methods: The GBM U-87 cell line was cultured on the chorioallantoic membrane of chicken eggs. After 12 days, cells were isolated and processed with H&E and immunohistochemical methods. The proliferative activity of GBM was established on the basis of mitotic and Ki-67$^+$ cells index. Apoptotic index was estimated by the Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling (TUNEL) method. Results: The biological activity of tumor tissue cultured in ovo corresponds to that of primary glioblastoma multiforme. Conclusion: GBM in in ovo model can be successfully applied in oncological studies.*

Glioblastoma multiforme (GBM) may occur as a result of uncontrolled glial cell proliferation as well as of lack of balance between their division and cell death. Many studies focused on glioblastoma multiforme biology and anticancer drug efficacy are conducted *in vitro*. Undoubtedly, there are some limiting factors of those experiment, including selection of established cell lines in the direction of desired characteristic (*e.g.* expression of specific genes, as well as characteristic morphological and functional features), which is achieved by serial passages. Moreover, during controlled growth, tumor cells grown in monolayer adapt to *in vitro* conditions. Monolayer culture consists of cells generally growing in a nutrient-rich liquid environment with plenty of oxygen, and they form genetically and phenotypically homogeneous cell populations. Tumor cells growing in such conditions lack the architectural and cellular complexity of *in vivo* tumors, which include stromal components, vasculature and inflammatory cell infiltration. So, it is impossible to recreate the complex interaction between the tumor and its host in *in vivo* conditions (1-4). The influence of antitumor drugs on glioblastoma cell activity can be successfully investigated on animal model (mouses and rats), and recently on *in ovo* models (5-6). This type of research is possible the only one with precise knowledge of biology of tumor cells growing in such conditions. To consider an *in ovo* model as a reliable tool for estimation of the anticancer drug efficacy, the biological activity of tumor growing *in ovo* conditions should be comparable to the spontaneous ones. Information about glioblastoma multiforme biology on *in ovo* models are limited. Most data concern the influence of anticancer drugs on expression profile of genes involved in angiogenesis. There is lack of information about the biological activity of tumor cells, growing in such experimental models. If the biological activity of such tumor, determined by proliferative and apoptotic indices, is similar to the spontaneous one, it confirms the utility of glioblastoma multiforme *in ovo* models in oncological research.

Correspondence to: Kaja Urbańska, Division of Histology and Embryology, Department of Morphological Sciences, Faculty of Veterinary Medicine, Warsaw University of Life Sciences, Nowoursynowska 159 Street, 02-776 Warsaw, Poland. E-mail: kaja.urbanska@onet.eu

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Materials and Methods

Cell culture. Human glioblastoma multiforme cell line U-87 MG (ATCC, No HTB-14) was incubated in Sanyo CO_2 Incubator (SANYO Electric Biomedical Co., Ltd., Osaka, Japan) under standard conditions (37˚C, 5% CO_2, 95% humidity), in Dulbecco's Modified Eagle's Medium (Sigma Aldrich Chemical Co., St. Louis, MO, USA) with the addition of inactive fetal bovine serum (10%

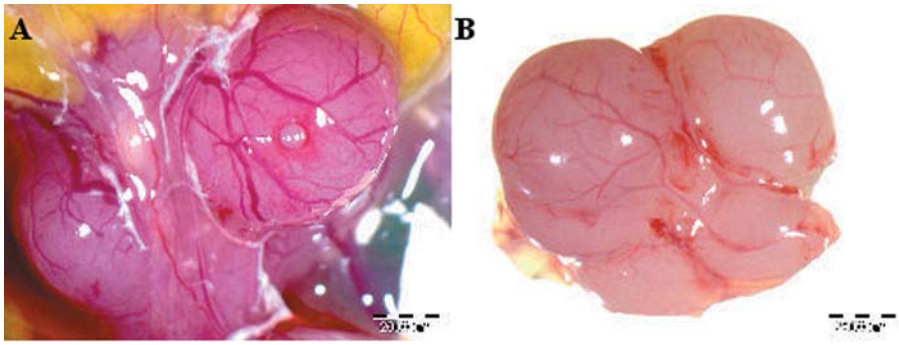

Figure 1. *Macroscopic view of glioblastoma multiforme tumors cultured on CAM (A) and after its isolation (B).*

v/v) (Sigma Aldrich) and antibiotics (penicillin (50 U/ml) and streptomycin (50 μg/ml) (Sigma Aldrich). Before transplantation to chicken embryos, the cell culture was treated with trypsin and EDTA (0.25% v/v, Sigma Aldrich). Then, cells were centrifuged (1,200 rpm, 5 min) and dispersed in culture medium in a concentration of 5×10^6/20 μl per egg.

Chicken embryo culture. The experiments were performed on 30 fertilised chicken eggs of *Gallus gallus f. domestica* meat race (Ross 308). The eggs were incubated in the incubator ALMD-1N3-7 (F.H.U. Waleński; Poland) with an automatic egg rotation system (one full rotation per hour) at 37˚C and 70% humidity.

Implantation of GBM cells on the chorioallantoic membrane (CAM) of chicken eggs. The process of implantation of GBM was performed at 6th day of the eggs incubation in laminar hood under sterile conditions. A 0.5-cm^2 hole was cut in the egg's shelf after its cleaning with potassium permanganate (Hasco Lek, Wroclaw, Poland). The internal parchment membrane of the air chamber was dissected and small silicone ring was placed on the blood vessels area and 5×10^6 tumor cells were dispersed in a 20-μl drop of medium placed into the silicone ring. Then, the hole in the egg's shell were protected by permeable plaster (Polopor, 3M Vicoplast S.A., Wroclaw, Poland) and eggs were moved to the incubator (37˚C and 70% humidity).

Tumor isolation. After the 18^{th} day of incubation, the chicken were terminated and the tumors were isolated (Figure 1A,B) and fixed in 4% buffered formalin. According to the Balke *et al*. (7) protocol only tumor with minimum 2 mm diameter, with visible area of vascularisation were analysed. In the case of few tumor formation in one egg, the tumor with the highest diameter, located the nearest silicone ring was choosen for future analysis.

Histological slide preparation and immunohistochemical analysis. After the fixation in 4% buffered formalin, samples were embedded in paraffin, cut into 4-μm sections, and stained with haematoxylin and eosin (H&E) and were immunohistochemically processed with anti-Ki-67 antibody and the Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling (TUNEL) method.

The tumor's proliferative activity was estimated by their proliferation index (PI) and mitotic index (MI).

The assessment of PI was based on Ki-67 protein expression in GBM cells. Ki-67 expression was determined by immunohistochemistry using anti-Ki-67 antibody, clone MIB-1 (Dako, Glostrup, Denmark). All immunohistochemical procedures were performed according to the manufacturer's protocol. Endogenous peroxidase was blocked by 5 min. incubation in H_2O_2 solution. Antigen unmasking was performed by using microwave (two cycles: 7 and 5 min., in citrate buffer, pH 6.0; 700 W). Sections were allowed to cool for 20 minutes, rinsed with TBST and then incubated with primary antibody, diluted 1:50, for 1 h at room temperature. Dako REAL™ EnVision™ Detection System, Peroxidase/DAB+, Rabbit/Mouse was used as a secondary antibody. PI was defined as the number of Ki-67-positive glioblastoma multiforme cells in 10^3 tumors cells. The marginal part of each section was excluded from analysis. Mitotic index (MI) was assessed as the mean number of metaphase and anaphase nuclei in 10 visual fields in triple counting (×400). The index was counted 3 times and average number was estimated in slides stained with H&E method.

The apoptotic activity of glioblastoma multiforme cells was defined as apoptotic index (AI). Apoptotic cells were detect by TUNEL method using ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Merck Millipore Headquarters, Billerica, MA, USA), according to manufacturer protocol. AI was defined as the percentage of apoptotic cells or apoptotic bodies in 1000 tumors cell population, without marginal areas of the tumors.

Results

In specimens from tumors growing on *in ovo* conditions U-87 cells showed a polymorphism regarding their size and shape. The atypical mitotic figures and giant cells were observed (Figure 2A and B). In all tumors blood vessels network was observed, however newly-formed capillaries did not reveal glomerular-shaped vessels (Figure 2C). Granulocyte and lymphocyte infiltrations were observed. In some cases apoptotic bodies and apoptotic cells were seen (Figure 3D). In all cases necrosis was rarely seen. There was no evidence of pseudopalisades around these necrotic foci.

Expression of Ki-67 protein was only observed in nuclei of glioblastoma multiforme cells, however the intensity of

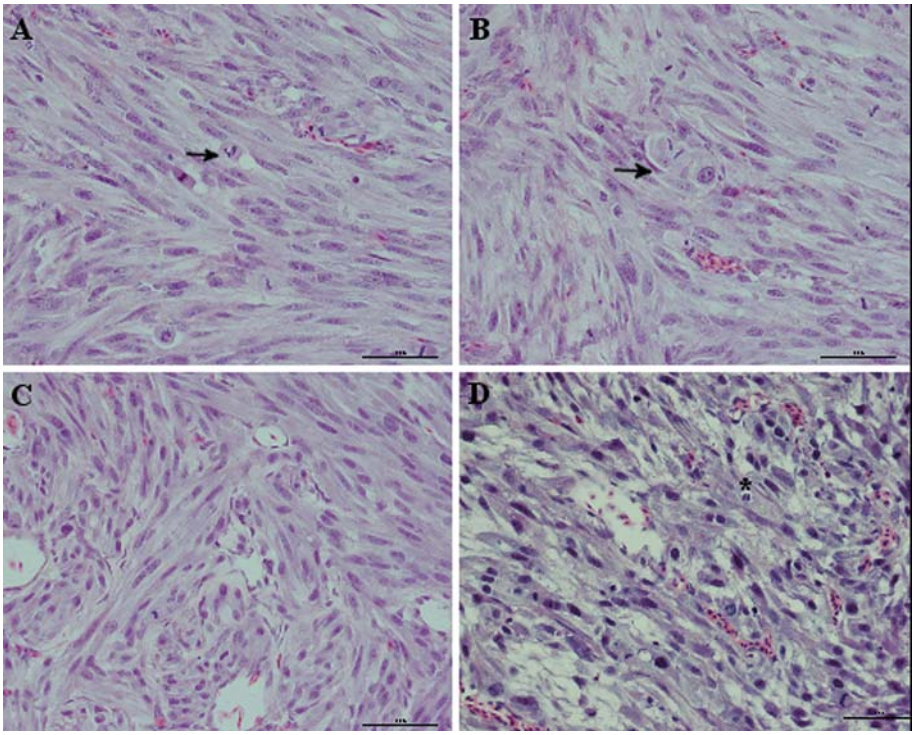

Figure 2. *Glioblastoma multiforme histology: (A) Mitotic figures (arrow), (B) giant cell (arrow), (C) blood vessels, (D) apoptotic cell (asterix).*

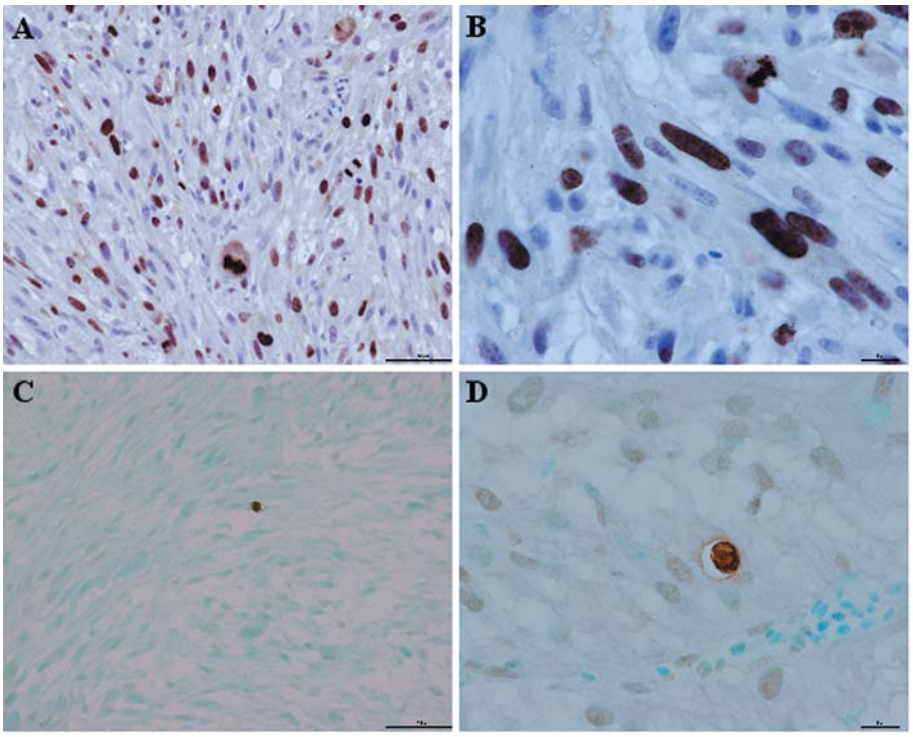

Figure 3. *Ki-67 expression in glioblastoma multiforme cells cultured on in ovo model (A, B) and apoptotic cells, TUNEL method (C, D).*

immunohistochemistry reaction differed between individual cases (Figure 3A and B). In some tumors, that reaction was very strong, and the morphological details of the nucleus were difficult to recognize. In other cases, those details were distinct. Tumor cells cultured on *in ovo* model had high proliferative activity, which exceed 20% in all cases, ranging from 21.9-36.1% (mean 28.72%±0.85). In 10 cases PI was 25-30% (10/20 cases). In 6 gliomas, the percentage of Ki-67$^+$ cells exceeded 30%. Only in 2 cases Ki-67 index was less than 25%.

The range of MI values was 4.00-12.33 (mean 8.54±0.48). In 12 cases the mitotic activity was estimated between 5-10. In 6 gliomas MI was very high (>10). In 2 tumors, MI did not exceed 5.

The TUNEL staining method showed a positive reaction in glioblastoma cell nuclei and in apoptotic bodies located among tumor cells or fagocyted by macrophages (Figure 3C and D). In cells located in small necrotic foci a positive cytoplasmic staining was seen, however those cells were excluded from the analysis. The AI was 0.7-2.4% (mean 1.12%±0.09). In most cases (10/20) apoptotic cells ranged between 0.5-1%. In one case the AI exceed 2.00%.

Results of immunostaining are summarized in Table I.

Table I. *PI, MI and AI in particular cases of glioblastoma multiforme cultured on in ovo model.*

Case number	PI (%)	MI	AI (%)
1	28.40	8.90	0.90
2	27.20	12.33	1.20
3	36.00	10.03	0.90
4	28.60	8.30	0.80
5	26.55	10.80	1.30
6	24.10	7.77	0.80
7	25.05	10.37	0.80
8	26.40	9.73	2.40
9	36.10	10.00	1.10
10	26.70	7.13	1.00
11	33.95	11.60	1.10
12	26.80	7.83	1.20
13	28.10	8.30	1.00
14	27.20	9.17	1.10
15	29.70	7.00	1.30
16	32.10	6.30	1.90
17	32.00	9.00	0.80
18	31.40	4.00	1.10
19	26.10	8.00	0.70
20	21.90	4.23	1.00

Discussion

Most studies focused on proliferative and apoptotic potential of glioblastoma U-87 cells have been performed on *in vitro* cultures. In signaling pathways activation and availability of the anticancer drugs to the tumor cells and to tumor microenvironment factors (extracellular matrix, partial pressure and blood vessels) are involved. The ion-trapping phenomenon is also well-known. It results from extracellular matrix acidic pH and causes a decrease of drug activity in *in vivo* conditions (8). The results of *in vitro* experiments have a significance cognitive value, but they also have some limitations, especially regarding bioavailability and bio-distribution of chemotherapeutic drugs. Although the *in ovo* model is used in experimental oncology research, to date there are no data about biological behavior (defined as proliferative and apoptotic activity) of tumors growing in such conditions, including in GBM (5, 6). If the biological activity of tumor growing on CAM will be similar to the spontaneous one, the *in ovo* model can be considered a reliable tool in oncological researches. Lack of these data render results of most experiments questionable. Certainly, results of such experiments do not fully describe the cell reaction occurring *in vitro*, however *in ovo* conditions are closer to *in vivo* reality. Because of that the estimation of proliferation and apoptotic activity of glioblastoma multiforme cells on *in ovo* model has some unique potential applications. There is only one publication regarding percentage of Ki-67$^+$ in glioblastoma cells line received after tumor cells inoculation on CAM. Strojnik *et al*. have compared proliferative potential of U-87 cells xenografts growing *in ovo* and in rats brains (5). The authors also calculated the Ki-67 index in monolayer and in spheroids. Ki-67$^+$ cell fraction was very high in all experimental systems, especially in glioma cells inoculated to rodent brains (75-80%). In our study, mean Ki-67 index in tumors growing *in ovo* amounted to 28.72% which corresponds to 30% as mean value reported by (5). Moreover, the percentage of Ki-67$^+$ cells growing *in ovo* is similar to results obtained by (9) for spontaneous glioblastoma multiforme.

The tumor specimens taken during surgery can also be inoculated on CAM (10-11), however, such cultures can not be kept longer than 6 days because CAM, which supplies nutrients, progressively dries with concomitant damage to blood vessels. Moreover blood vessels do not invade the transplants. It leads to death of transplated cells (11). Balciūniene *et al*. have shown Ki-67$^+$ cells only in few tumors growing in such conditions (11). That was caused by an increase of necrosis as a results prolongation time of tumor incubation. In these cases the proliferation of tumor cells was limited. Unfortunately, the authors did not present any Ki-67 numeric data for those cases.

The present study was conducted on tumor growing 12 days on CAM. Proliferation index was estimated as a percentage of glioblastoma multiforme Ki-67$^+$ cells, as well as mitotic index. Those proliferative indices are used in assessment of effectiveness of surgical procedures as well as

pharmacological and radiological anticancer therapies (12). In our study gliomas PI was ranging 21.9%-36.1% (mean=28.72%). Ki-67$^+$ cells were uniformly-distributed in the tumor tissue, except from marginal zone, where the fraction of positive cells was higher. The intensity of nuclei stain was different. No unspecific staining nor any positive reaction in the cytoplasm were observed in neoplastic cells. The ranges of PI values in our study are similar to Ki-67 expression in GBM growing on CAM (5), as well as in spontaneous glioblastoma multiforme. It confirmed the utility of the *in ovo* model in glioblastoma multiforme studies. However Torp observed an increase of Ki-67$^+$ cells in perivascular area (13). Other studies have shown, that pseudopalisading cells possess 5%-50% less proliferative capability compared to other tumor areas (14). In all examined glioblastomas growing on the *in ovo* model such areas were not observed, so comparisons of this sort could not be done.

Glioblastoma multiforme has the highest proliferation index among the tumors of astrocytic origin (15, 16). More than 98% of diagnosed glioblastomas are primary tumors (17). Their proliferation indices range between 25-25.60% and 25-29.40% in adults and children, respectively (18-20). Most of glioblastomas cause death within 12 months after diagnosis. Long-term survival of patients suffering from glioblastomas are rarely noted. The Ki-67 index is lower in patients with ≥5 years survival rate (≤12%) (21, 22). In our study mean PI was 28.72%. This indicates that proliferative activity of glioblastoma cultured on CAM is similar to de novo glioblastoma development, which causes death within 12-16 months after diagnosis. Such cases are the most common. However, some authors have shown, that Ki-67 index of spontaneous glioblastoma multiforme is very low (23), while in other publications the proliferation index of these tumors was very high and exceeded the mean tumors PI value observed in our study. Glioblastoma multiforme with PNET component has an extremely high Ki-67 index (nearly 100%). Metastatic tumors also possess a more aggressive behavior (defined as Ki-67$^+$ cells), which causes higher cell resistance for radio- and chemotherapy (24). However, primary gliomas with PI ranging from 0-0.9% Ki-67$^+$ cells were a surprise (13, 25-26), as their proliferative potential is not consistent with WHO characteristics. Probably the wide range of Ki-67 values can be explained by heterogeneous phenotype of glioblastoma resulting from clonal selection of its cells. This can explain existence of some differences in glioblastoma proliferative potential, even in one case (27). This indicates, that Ki-67 index is useful tool to define tumor biological behavior (28-29). PI obtained in our study are similar to PI estimated by Burton *et al*. (30), who calculated PI in long-term GBM survivors (≥36 months) compared to typical survivors (≤18 months) but the boundaries of these ranges (3.8-85.2%; 6.2-69.9%) differ from that obtained in our study (21.9-36.1%) for tumors growing on *in ovo* model. Such high divergences in PI extreme values received by Burton *et al*. (30) can result from a wide variety of glioma cells, as well as Ki-67$^+$ cells counting from all areas of tissue sections, including marginal zones and including into study very small tumor sections (containing less than 1000 neoplastic cells). In other publications also noted the high Ki-67$^+$ glioblastoma multiforme cells divergences (26, 29, 31). It can result from different methods of assessment of Ki-67 antigen expression (manual counting *vs*. computer-automated estimation) (32). We have observed, that similarly to spontaneous tumors, the PI of glioma cultured on CAM differ between individual cases, however in any of those tumors difference was not higher than 15%. Certain clinical cases of gliomas with PI differences higher than 15% were noted (33). The relatively constant value obtained for tumors growing *in ovo* can result from providing the same culture conditions for all tumors. PI is of prognostic value in human medicine and facilitates prognosis prediction for individual patients (34, 35), however some authors have not confirmed it (14).

Mitotic activity is fundamental in histopathological grading of human astrocytomas (36). In our study the MI of GBM cultured *in ovo* was ranging 4-12.33 (mean=8.54), similarly to results obtained to the spontaneous ones (37). Surprisingly, mean mitotic activity of our tumors was lower than that observed in GBM patients with average survival rate ≥5 years (8.54 *vs*. 9.8) (38).

Necrosis is the most common type of malignant tumor cell death, however it is possible that a large percentage of tumor cells die *via* apoptosis (39). The evidence of impaired apoptosis as a prerequisite for tumorigenesis and ability of tumor cells to avoid this type of death is well-known (40-41). Nevertheless the significance of apotosis in oncogenesis is still poorly-understood. According to one perspective, apoptosis of tumor cells can impact on the loss of their immortality. According to the opposite theory, apoptosis stimulates clonal development of tumor cell population (42). A high frequency of apoptosis is observed in spontaneously-regressing tumors, as well as in tumors treated with cytostatic drugs. This kind of process is important for studies developing new anticancer drug to render self-elimination of tumor cells easier (39). The estimation of apoptotic index (AI), similarly to PI, is a significant indicator of tumor growth and it has significant prognostic value (43).

Cells in the central nervous system exhibit differences regarding their resistance to apoptosis. The most vulnerable ones are neurons, followed by oligodendrocytes, astrocytes and endothelial cells with microglia being the most resistant. During malignant progression most of the cell types acquire high resistance to apoptosis-inducing agents. Cells at the marginal zones of glioblastoma multiforme tumors are less sensitive to apoptosis which results in frequent recurrences. The apoptotic index of glioblastoma multiforme is not high. There exist many mechanisms that allow for tumor cells of

astrocytic origin to avoid apoptosis and continue their growth (44). There exist publications in medical oncology, that investigate glioblastoma multiforme cell apoptosis. Most of them are focused in establishing the relationship between the apoptotic and proliferation activity of glioblastoma multiforme cells (45, 46). Increase of apoptosis in relation to increase of histological grade of astrocytic tumors was confirmed by Sipos *et al*. (47). Carroll *et al*. (44) found the highest apoptotic index in glioblastoma multiforme compared to anaplastic astrocytoma and astrocytomas with lower grades of malignancy. The same correlations have been shown by Sarkar *et al*. (48). Glioblastomas had the highest AI, among all examined CNS tumors, however the AI values were only 0.7% and 0.84% (44, 48). Unfortunately, some other authors have not confirmed these correlations (49). In the present study AI of glioblastoma cells cultured *in ovo* ranged from 0.7-2.4% (mean=1.12%). Obtained results are similar to Caroll *et al*. (44), Takekawa *et al*. (49), Korshunov *et al*. (50), and Schiffer *et al*. (51), who have shown, that AI of spontaneous glioblastoma were less than 3.00%. AI of the tumors inoculated on CAM are also similar to results obtained by Mellai *et al*. (33) (AI=0.00-1.40%) and Korshunov *et al*. (52) (AI=0.73%). It is believed, that higher apoptotic activity of glioblastoma multiforme (compared to other CNS tumors) is evidence of high proliferative activity of their tumor cells (53). There are only few publications about glioblastoma multiforme cases with high AI values (12.80%, 32.20% and 8.60%) (45, 47), which did not correspond to our results. According to some data, the intensity of apoptosis in glioblastoma multiforme does not always correspond with tumor growth inhibition (47). In our results glioblastoma cells ongoing apoptosis, as well as apoptotic bodies were randomly dispersed in whole tissue sections. The positive cytoplasmic reaction in cells situated in necrotic foci was also occasionally noted, but these cells were not included in analysis. In human glioblastoma multiforme with AI less than 0.50%, most of apoptotic cells are located near necrotic zones. In cases with AI >0.5% positive cells are dispersed in whole sections (50, 52). The same results were obtained by Takekawa *et al*. (49), who noted the high AI (mean 6.74%) in pseudopalisades. In other parts of the tumor apoptotic cells were occasionally found (mean=1.07%). The high AI in pseudopalisades was also described by Brat *et al*. (14). It indicates that apoptosis is one of the factors influencing formation of pseudopalisades.

The biological characteristics of glioblastoma multiforme cultured on an *in ovo* model, defined as its proliferative and apoptotic activities, correspond to those of primary spontaneous tumors. GBM on an *in ovo* model can be applied in oncological studies, however expression of the major proteins involved in those processes should further analyzed.

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