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Use of Chicken Embryos as an Angiogenesis Model for Central Nervous System Malignant Tumor Research

Nurcan UMUR[1], Seren Gulsen GURGEN[2], Hayrunnisa YESIL SARSMAZ[3], Ahmet Sukru UMUR[4]

[1]Manisa Celal Bayar University, School of Vocational Health Service, Department of Molecular Biology, Manisa, Turkey
[2]Manisa Celal Bayar University, School of Vocational Health Service, Department of Histology and Embryology, Manisa, Turkey
[3]Manisa Celal Bayar University, Faculty of Health Science, Department of Histology and Embryology, Manisa, Turkey
[4]Manisa Celal Bayar University, Faculty of Medicine, Department of Neurosurgery, Manisa, Turkey

Corresponding author: Ahmet Sukru UMUR ✉ umuras@yahoo.com

ABSTRACT

AIM: To demonstrate the usability of chicken chorioallantoic membrane (CAM) as an angiogenesis model for the development and treatment of malignant tumors of the central nervous system.

MATERIAL and METHODS: A fresh tumor tissue piece taken from Glioblastoma patients, a malignant tumor of the central nervous system, was transferred to the CAM of chicken embryos and left to incubate in the incubator and their development was monitored. After examining the results of the study macroscopically, CAM tissue samples were evaluated both histochemically and immunohistochemically in terms of angiogenic factors VEGF (Vascular Endothelial Growth Factor), bFGF (basic Fibroblast Growth Factor) and PDGF (Platelet Derived Growth Factor).

RESULTS: According to histochemical findings obtained from our study when compared with control embryos, blood vessels, fibroblast count and inflammatory infiltration were observed more in the tumor transplanted groups, especially in the tumor-developing CAM region. There was also intense pleomorphism and marked hypercellularity in the cells. In our immunohistochemical findings, it was determined that bFGF, PDGF, VEGF staining intensities were higher in tumor transplanted groups compared to control groups, and this elevation was more pronounced in the tumor-developing region.

CONCLUSION: As a result, it has been shown that the chicken embryo CAM model may be a suitable in vivo model for cancer angiogenesis studies. The protocol we created in this study will be a source for projects related to the use of therapeutic agents in cancer angiogenesis.

KEYWORDS: Chorioallantoic membrane (CAM), Glioblastoma, Angiogenesis

INTRODUCTION

Cancer is an ongoing pathological condition in the form of uncontrolled or abnormal growth and proliferation of cells, which occurs as a result of a malfunction in the mechanisms regulating the cell cycle. Today, it is the second leading cause of death after cardiovascular diseases (16). The formation of new blood vessels is needed to provide oxygen, nutrients and growth factors required for tumor tissue growth, invasion and metastasis. New vessel formation, called angiogenesis, is one of the most important mechanisms in cancer progression (6,10,11). Today, many in vitro and in vivo study models have been proposed for cancer research. Although in vitro models provide important information regarding the process of tumor angiogenesis, they cannot fully demonstrate what the in vivo effect will be. The chicken chorioallantoic membrane model (CAM) is one of the major in vivo models that can be used for tumor growth and angiogenesis, virus research, new drugs, and therapeutic studies. CAM is a well-vascularized extraembryonic membrane that functions for gas and nutrient exchange and waste removal for the growing chicken embryo. It is accepted as a study model

Nurcan UMUR : 0000-0001-6593-8751
Seren Gulsen GURGEN : 0000-0002-5514-1404
Hayrunnisa YESIL SARSMAZ : 0000-0002-9790-1723
Ahmet Sukru UMUR : 0000-0002-9708-7346

that is easy to apply, simple, inexpensive, reproducible and allows the observation of angiogenic response for experimental studies (7,13,30,33,39). At the same time, it has the characteristics of reflecting the morphological and biological features of the tumor for cancer research, evaluating invasiveness, tumor progression, and enabling the search for new drugs. In these studies, it is an important advantage that chicken embryos naturally have insufficient immune system and do not show tissue transplant rejection reaction until the 18th day (8,9,15,25,27,28).

Glioblastoma is the most common type of tumor of the central nervous system (CNS), which has an extremely poor prognosis as well as a low survival rate despite advances in surgery and clinical neuro-oncology. It is very heterogeneous in terms of its biological and morphological features and is widely studied (24). Although there are studies on tissue analysis or cell culture of factors related to angiogenesis in Glioblastoma in the literature, there are limited studies using the CAM model in vivo (28,32,41). In vivo models of glioblastoma depend on the inoculation of glioma cells or cell lines into the brain of certain experimental animals to form tumors. In these models, tumor penetration and invasiveness are inadequate and growth is variable. Hence, exact morphological data cannot be acquired. At the same time, it does not fully reflect the interaction between tumor and host, correct invasion processes, vascularity, gene expression profile, and stroma interactions that occur in humans (7,13,30).

In the light of this information, we aimed to demonstrate the usability of the chicken embryo CAM model as an angiogenesis model for CNS malignant tumor development and treatment studies. Thus, tumor tissue taken from Glioblastoma patients was transferred to the CAM and its invasiveness was observed. Changes in the membrane were examined by both histochemical and immunohistochemical methods using angiogenic markers.

MATERIAL and METHODS

This study was approved by Manisa Celal Bayar University Health Sciences Ethics Committee (Decision no: 20.478.486), supported by Manisa Celal Bayar University Scientific Research Projects Coordination Unit with project number 2019/096.

Embryo Chicken Eggs

In our study, 200 fertile, daily, white Leghorn type chicken eggs with specific pathogen-free (SPF) ranging from 50 to 72 g obtained from Bornova Veterinary Control Institute were used. Ensuring the continuity of the embryos and cultivating the tumor tissues at the desired times was done in the incubator in the Neurosurgery Department of the MCBU Faculty of Medicine.

Malignant Tumor Tissues

Five patients who were admitted to the MCBU Hafsa Sultan Hospital Neurosurgery clinic, were clinically and radiologically diagnosed with Glioblastoma and decided to be operated were determined. Consent form was filled by the patients and their consent was obtained. A piece of 1 cm^3 was taken from the tumor tissue removed during the operation into saline and transferred to the incubated eggs. The diagnosis of glioblastoma was confirmed by the pathologists.

Incubation of Embryo Chicken Eggs

Eggs to be transplanted with tumor tissue (n=30) and eggs without any treatment (n=10) were incubated in the incubator. Care was taken that the eggs were not cracked, their shells were clean, and that they were not shaken too much when placed in the incubator. The incubator was kept at a constant temperature of 37-37.5 ºC and humidity of 50-60%. During incubation the eggs were turned. Incubation was continued until the 7th day when the CAM was fully developed according to Hamburger and Hamilton staging and ensuring the continuity of the embryos (Figure 1).

Transplantation of Tumor Tissue to the Chicken Embryo Chorioallantoic Membrane

After the shells of the eggs removed from the incubator on the 7th day were cleaned with alcohol, a rectangular 1.5x1.5 cm window was opened on the non-pointed side of the egg shell with sterile surgical instruments. Tumor tissues, which were cut into small pieces of approximately 0.2x0.2 cm, were transferred to the CAM. Afterwards, the opened window was covered with a plaster and placed back in the incubator and no longer rotated. The first hatching day of the eggs was called day 0 and was followed in the incubator until day 17 (Figure 2).

Removal of CAM and Developing Tumor Tissue from Chicken Embryo

In order to obtain the CAM of Embryos on day 17, the embryo was first removed. For this, the embryo was removed after the egg white was poured out. CAM samples were taken from areas that developed tumors and were distant from the tumor, and placed in 10% formalin solution (Figure 3).

Histological and Immunohistochemical Analyzes of the Tissues

Histochemical Staining

After the tissues were fixed for 24 hours, they were washed in running tap water for a night in order to remove the fixatives.

Figure 1: Incubation of Embryo Chicken Eggs in an incubator.

Figure 2: Tumor tissue transplantation into chicken CAM.

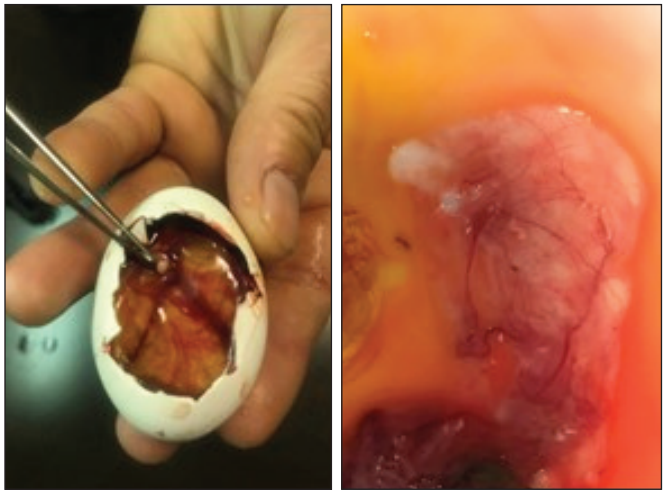

Figure 3: Removal of CAM that has developed tumor tissue.

For the purpose of dehydration, it was passed through ethyl alcohol series increasing from 60% to 100% for five minutes. After being kept in xylene-alcohol for five minutes, two changes were kept in xylene for seven minutes for transparency. After applying xylene-paraffin for ten minutes in an oven at 60 ºC, the tissues were embedded in paraffin blocks. For both histochemical and immunohistochemical examination, 5µ serial sections were taken from the blocks by means of a rotary microtome. For histochemical analysis, some of the sections were stained with Hematoxylin-Eosin in accordance with the routine protocol and covered with entellan.

Indirect Immunohistochemical Staining

The sections were cleared with two changes of xylene for eight minutes, after an overnight stay in an oven at 37 ºC and kept at 60 ºC for an hour. Then, rehydration was provided with alcohol series decreasing from 100% to 60% for three minutes and kept in distilled water twice for five minutes. Sections confined with an immunohistochemistry pen (Dakopen) were kept in 2% trypsin solution at 37 ºC for 10 minutes and then washed three times with PBS phosphate buffer solution for five minutes in order to reveal antigenic sites. In order to inhibit tissue endogenous peroxidase, 3% H2O2 was administered for 15 minutes. After washing three times with PBS phosphate buffer solution for five minutes, the sections were kept in the blocking solution for ten minutes. After the blocking solution was removed from the tissue by washing with PBS, it was incubated in a humid chamber with the primary antibodies anti-bFGF (Rabbit polyclonal antibody, Santa Cruz, CA, USA), anti-PDGF (Rabbit polyclonal antibody, Santa Cruz, CA, USA), and anti-VEGF (Rabbit polyclonal antibody, Santa Cruz, CA, USA) for an hour at room temperature. All antibodies were diluted 1/100. Then, sections washed 3 times with PBS buffer solution were applied biotinylated secondary antibody and conjugated streptavidin-horseradish peroxidase for 20 minutes. AEC (3-amino 9-ethylcarbazole) chromogen was applied for 3-5 minutes in order to determine the visibility of the immunohistochemical reaction in which sections were washed with PBS buffer solution 3 times for 5 minutes. After the nuclei were stained with Mayer's hematoxylin, the sections were washed with distilled water for 10 minutes and covered with Mounting Medium. The immunoreactivity intensity of the sections examined at different times and by two different researchers, according to the brown staining intensity of the AEC chromogen, was evaluated under a light microscope (Olympus, Tokyo, Japan) and photographed.

Histological and Immunohistochemical Evaluation

In the staining, five areas were selected randomly at X400 magnification for each preparation, and the H score was calculated according to the intensity of the involvement and the percentage of uptake. Intensity of involvement was semi-quantitatively scored as 0 (0, no involvement), 1 (+, weak immunoreactivity), 2 (+ +, moderate immunoreactivity), 3 (+ + +, strong immunoreactivity). The percentage of uptake was scored as 1 (0-10%, focal), 2 (11-50%, regional), and 3 (51-100%, diffuse) by dividing the cells/structures with immunoreactivity to the total cells/structures. Intensity and amount scores for each area were calculated with the formula Σ Pi (i+1) (Pi = percentage of uptake, i = intensity of uptake). The results were summed to arrive at a single value for that area.

Statistical Analysis

SPSS 23.0 (SPSS Inc.; Chicago, IL, USA) package program was used to evaluate the data. In the analysis of the data, the focus was on the average ±SD values. ANOVA test was used because the numerical data were in accordance with

the normal distribution. Results with a P value less than 0.05 were considered statistically significant. Post hoc multiple comparison test (Tukey) was applied to understand from which group the difference was.

RESULTS

Histochemistry Results

The histological structure of the CAM region of control embryos was externally surrounded by cubic epithelial cells. Under the epithelium, fibroblasts and blood vessels were distinguished in the loose connective tissue (Figure 4A). Inflammatory infiltration with marked increase in the number of blood vessels and the number of glial cell nuclei was observed in the tumor-developing CAM region. There was intense pleomorphism and mitotic increase in cells. Regional concentrations of inflammatory cells were observed. (Figure 4B). Thickness increase was evident in the CAM region distant from the the tumor (Figure 4C1). Significant hypercellularity in the connective tissue of the membrane and an increase in blood vessels were remarkable. In addition, inflammatory infiltration was detected in the connective tissue (Figure 4C2) (Table I).

Immunohistochemistry Results

CAM tissue sections of control and tumor transplanted eggs were stained by indirect immunohistochemical method to examine the protein distributions of bFGF, PDGF, VEGF. The CAM tissue sections of the chichen, which were transplanted from the tumor, were divided into 2 groups as the region where the tumor tissue developed and the region away from it.

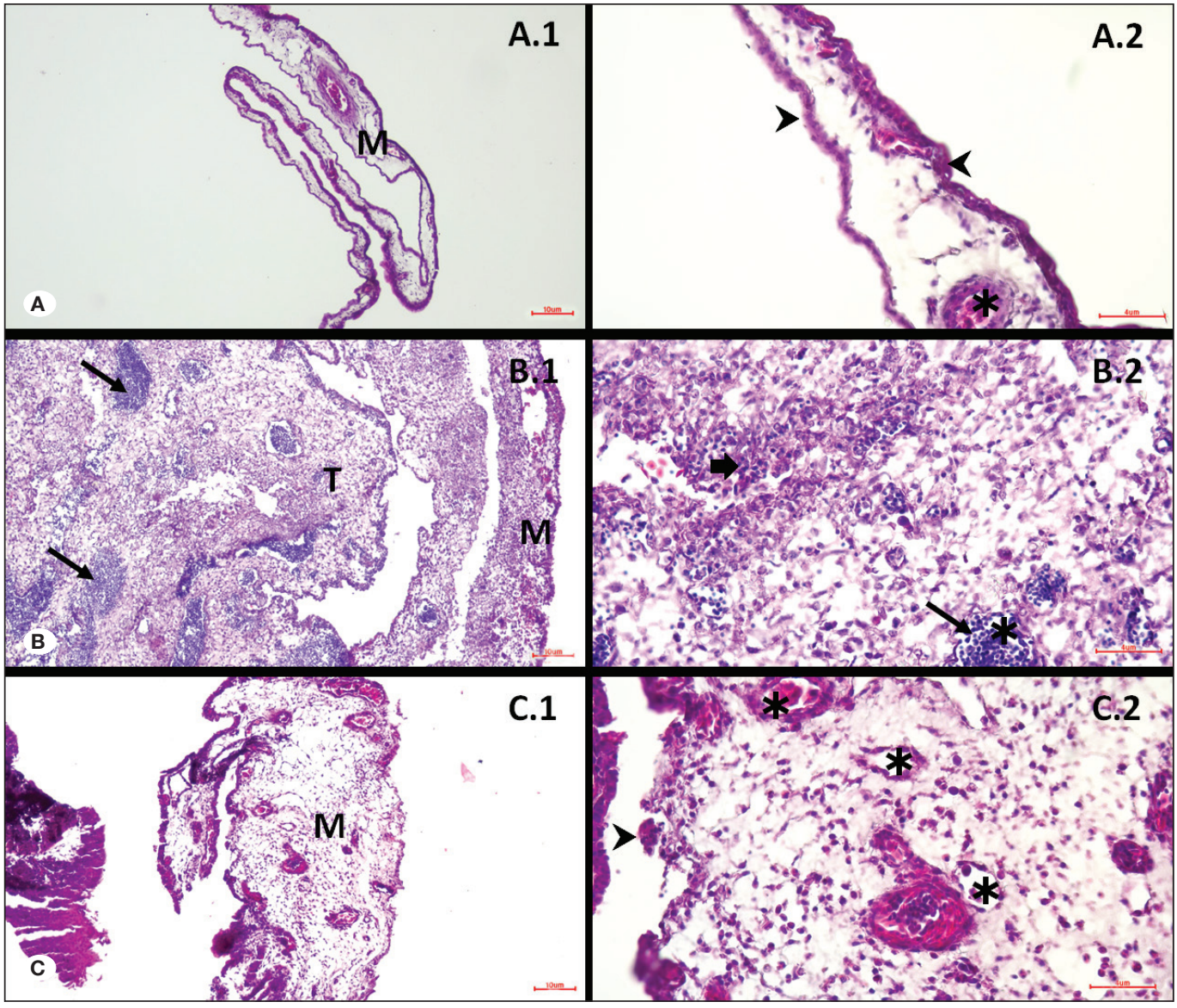

Figure 4: Histochemical Staining. Control group CAM region **(A)**, tumor transplanted group; tumor growing region **(B)**, distant region from tumor **(C)**. **M:** Membrane, **T:** tumor, →**:** Inflammatory infiltration, ➡**:** Hypercellularity, ✱**:** Blood vessel. (1) (x100) Bar = 10μm, (2) (X400) Bar = 4μm.

In the control group, a weak to medium reaction was observed in the epithelium immunostained with bFGF in the CAM region, while it was weak in the connective tissue (Figure 5A). In PDGF and VEGF immunohistochemistry stainings, moderate to strong immune reaction was observed in the epithelium and moderate in the connective tissue. It was noteworthy that especially PDGF and VEGF were higher than bFGF in the control group (Figure 5B, C).

Table I: Histochemical Findings of the Groups

Groups		Blood vessel	Fibroblast	Lymphocytic infiltration	Hypercellularity
Control Embryos	**CAM**	+	+	-	-
Tumor Transplanted embryos	**Group 1 (Tumor developed CAM region)**	++++	+++	++++	++++
	Group 2 (CAM region distant from the tumor)	+++	++	+++	+++

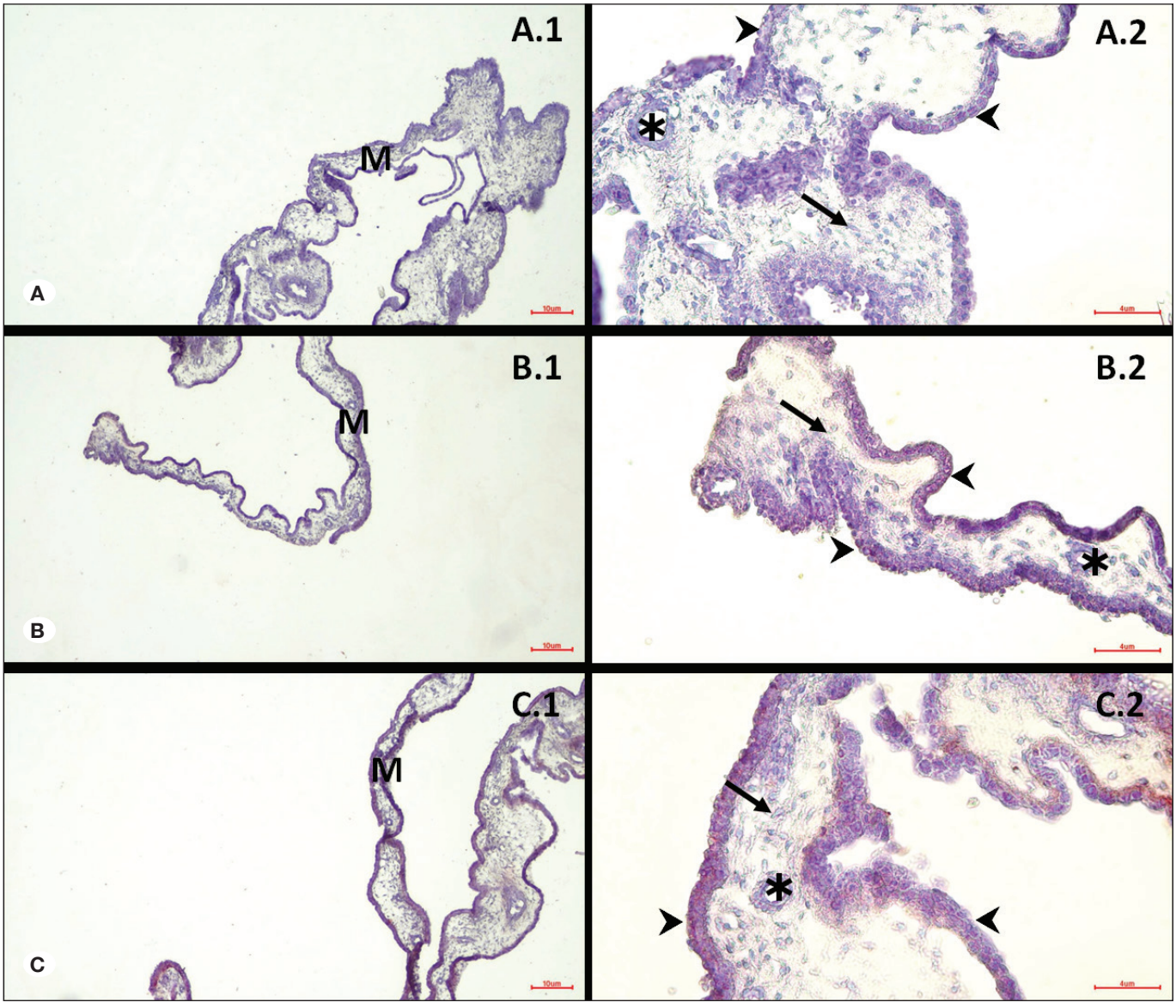

Figure 5: Immunohistochemistry staining of the control group CAM region. FGF **(A)**, PDGF **(B)**, VEGF **(C)**. **M:** Membrane ➤**:** Epithelium, ✱**:** Blood vessel,→**:** Connective tissue. (1) (x100) Bar = 10 µm, (2) (X400) Bar = 4µm.

FGF and VEGF immunostaining showed very strong expression in the membrane epithelium and connective tissue in the tumor developed region of the tumor transplanted group. It was noted that mostly infiltrative cells were strongly positive in the tumor tissue (Figure 6A, C). On the other hand, in PDGF staining, some infiltrative cells and hyaline granular bodies in the tumor tissue were strongly positive, while other cells stained moderately (Figure 6B). In the CAM region distant from the tumor, immunostaining of bFGF and PDGF was observed to stain weakly in the epithelium and strongly in the connective tissue (Figure 7A, B). In VEGF staining, the reaction was observed to be moderate in the membrane epithelium, while it was observed that the uptake was quite severe in the connective tissue (Figure 7 C).

When immunoreactivities were compared in all three groups, it was determined that staining intensities of bFGF, PDGF and VEGF, which are angiogenic factors, were found to be statistically significantly higher in tumor transplanted groups compared to control groups ($p<0.001$). It was found that the staining intensities of bFGF, PDGF, VEGF staining in the region where the tumor developed (Group 1) were higher than in the CAM region distant from the tumor (Group 2), and these increases were statistically significant ($p<0.001$) (Table I, Table II, Figure 8).

DISCUSSION

Cancer cells can multiply uncontrollably and metastasize. In this process, providing the necessary oxygen and nutrients

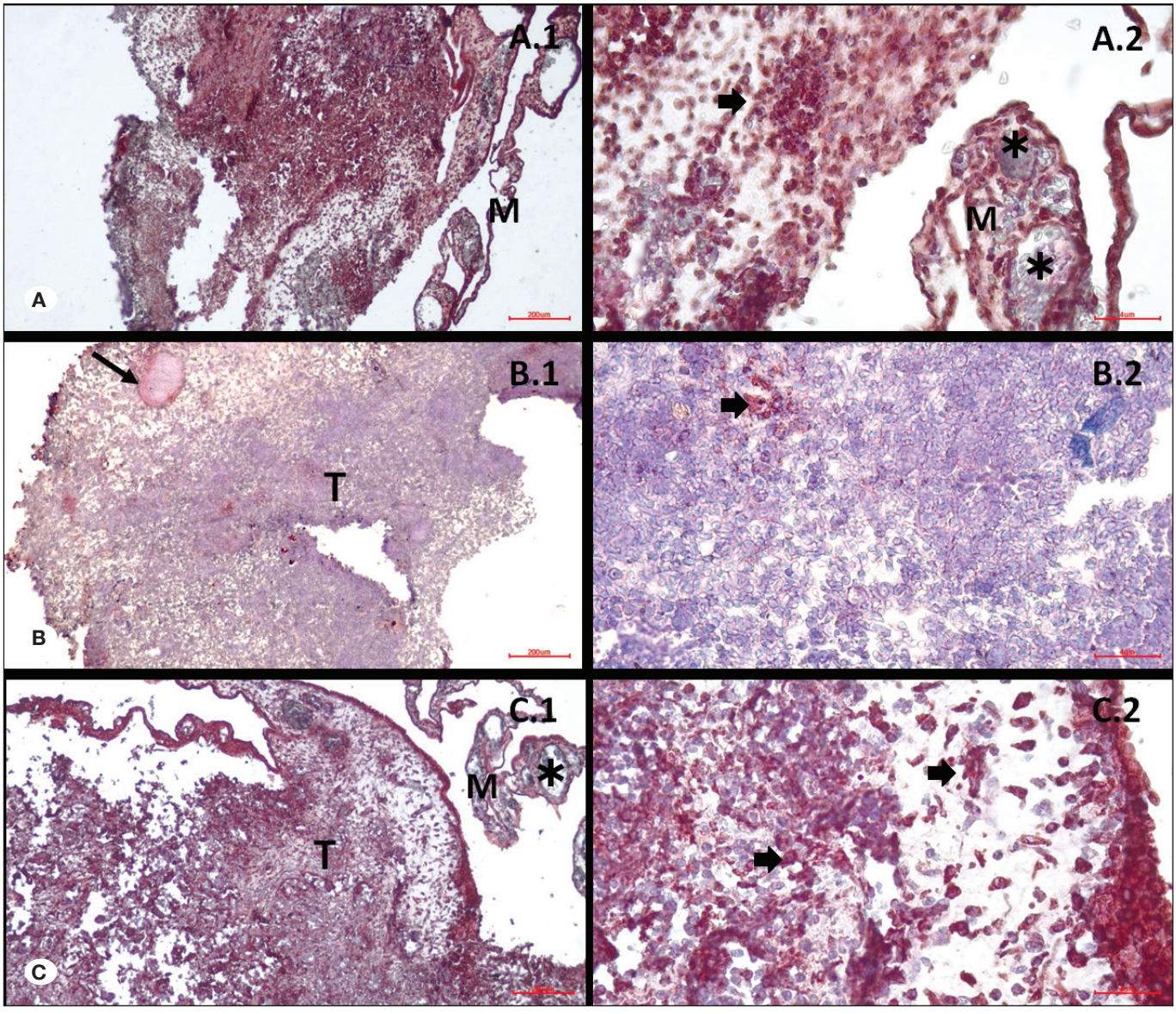

Figure 6: Tumor transplant group; Immunohistochemistry staining of the tumor-developing CAM region (Group 1). FGF **(A)**, PDGF **(B)**, VEGF **(C)**. **M:** Membrane, **T:** Tumor, **→:** Hyaline granular bodies, **➡:** Immune reaction positive infiltrative cells, **✱:** Blood vessel. (1) (x100) Bar = 10μm, (2) (X400) Bar = 4μm.

Table II: FGF, PDGF, VEGF HSCOR Levels in CAM Tissues of Control and Tumor Transplanted Groups

HSCOR		FGF	PDGF	VEGF
		Mean ± ss	Mean ± ss	Mean ± ss
Control embryos	CAM (Control)	96 ± 4.32	112.28 ± 4.82	130.85 ± 7.19
Tumor Transplanted embryos	Group 1 (Tumor developed CAM region)	260 ± 8.64	213 ± 6.48	298.28 ± 10.54
	Group 2 (CAM region distant from the tumor)	219 ± 6.48	195 ± 6.48	252 ± 6.48

**p<0,05 (Anova test).*

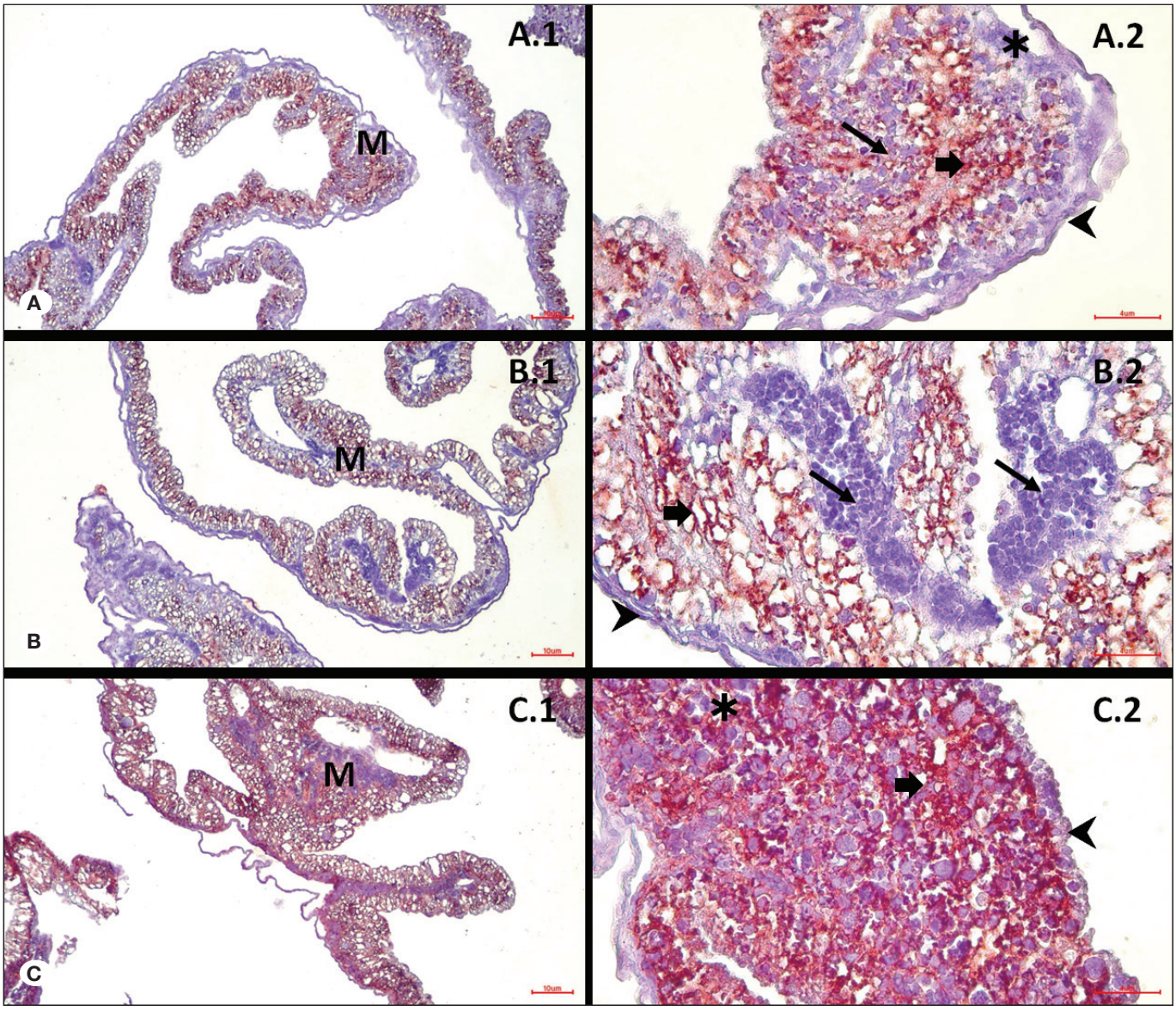

Figure 7: Tumor transplant group; Immunohistochemistry staining of the CAM region free from the tumor (Group 2). FGF **(A)**, PDGF **(B)**, VEGF **(C)**. **M:** Membrane, ➤**:** Epithelium, →**:** Inflammatory cells, ➡**:** Immune reaction positive infiltrative cells, ✱**:** Blood vessel. (1) (x100) Bar = 10µm, (2) (X400) Bar = 4µm.

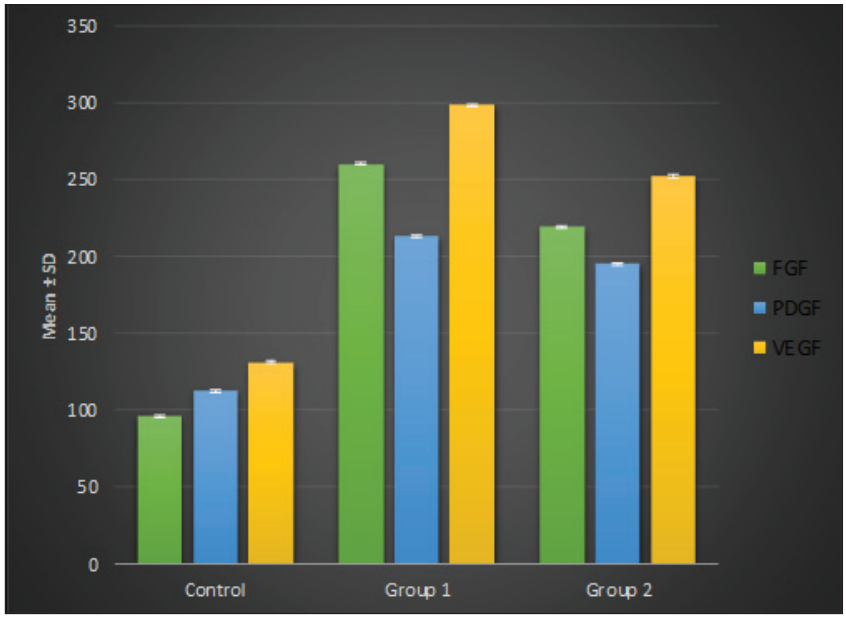

Figure 8: Morphometric data for bFGF, PDGF, VEGF staining. Data are expressed as Mean ± SD.

as well as removing wastes are provided by a new network of blood vessels called angiogenesis. The rate of angiogenic activity in a tumor is the result of the interaction between tumor cells, endothelial cells, and inflammatory cells. Angiogenic and antiangiogenic factors originating from these cells have both autocrine and paracrine effects. The transition of a tumor to an angiogenic phenotype depends on the change in the balance between angiogenic and antiangiogenic regulators. Angiogenic activity begins when angiogenic factors come to the fore due to various factors in the environment (5,23). In order for angiogenic activity to start, not only the increase in angiogenic factors, but also the effects of antiangiogenic factors must be overcome. Under normal conditions, antiangiogenic factors prevent angiogenic activity by protecting the vascular endothelium from stimuli. Some of these factors disappear completely when the tumor acquires angiogenic characteristics. The most important ones are Thrombospondin, Angiostatin, Endostatin etc. (21,26). After Folkman suggested in 1974 that stopping tumor angiogenesis might be a cancer treatment option, although the mechanisms of this phenomenon have been largely revealed today, there are still many questions to be answered (10). Today, CAM is used as an in vivo experimental model in cancer angiogenesis research to study molecules with angiogenic and antiangiogenic activity, various tumor types and their growth rate, angiogenic potential, and metastatic capacity (1,13,25,28). The main advantage of the CAM model over in vivo models is its low cost, simplicity, reproducibility, and reliability. In the developmental stage (ED 6-10), where tumor grafts are placed on the CAM, the conditions for tissue rejection have not yet been established, since the chick's immune system is not fully developed (22,35). However, it is also possible to observe nonspecific inflammatory reactions despite the immaturity of the immune system. Compared to mammalian models, where tumor growth takes between 3 and 6 weeks, tests using chick embryos are faster. 2 to 5 days after tumor biotic specimens implanted on the CAM surface, tumor grafts become visible and are fed by CAM-derived vessels. Finally, CAM does not require the procedure for obtaining ethics committee approval for animal experiments, because the chicken embryo is not considered a live animal until day 17 of development (7,28,29).

Glioblastoma is a type of cancer with an extremely poor prognosis and a very low survival rate, corresponding to Grade IV glioma. It is characterized by an intense and abnormal angiogenesis (24). In the histopathological structure of GBM, multinucleated highly proliferative cells, numerous blood vessel clusters forming necrotic areas and glomerular structures, and endothelial cell proliferation were observed (42). The best-known regulators of angiogenesis in Glioblastoma progression; FGF-2, VEGF, PDGF, transforming growth factor beta (TGF-β), hepatocyte growth factor (HGF), matrix metalloproteinases (MMPs), and angiopoietins. These angiogenic molecules are regulated by many mechanisms (eg, loss of tumor suppressor gene function, oncogene activation, hypoxic microenvironment). Studies have shown the presence of these molecules in a wide variety of cancer types, including glioblastoma, and sometimes by normal cells, and it has been shown that their increase is especially related to malignancy (4,29,31,36,42). Therefore, we preferred angiogenic factors bFGF, PDGF and VEGF antibodies to examine glioblastoma progression in the CAM model.

Hurst et al., Vogel and Berry, Klags-brun et al. conducted the first studies in this field in the literature. They demonstrated that different brain tumors can be transplanted into the CAM and tested its angiogenic capacity, showing that the most potent cells are glioblastoma and meningioma brain tumor cells (17,20,40). In the study of Karnofsk et al., the behavior of chicken, mouse and human tumor cells and tissues implanted on the CAM surface were compared. Furthermore, tumor growth, histological features, viability, and effects

on the original host after transplantation were examined (18). Kaufman et al. explained the changes in the chorionic epithelium at the site of tumor transplantation (19). According to the study of Hagedon et al. transplanted glioma onto CAM, tumors form a hemorrhagic, necrotic and edematous tumor tissue within two days via VEGF receptor-2 (14). Tereseviciute et al. transplanted Glioblastoma biotic specimens onto CAM and showed tumor growth after 7-9 days (38). The tumors were immunopositive for S100 protein, vimentin, glial fibrillary acid protein (GFAP), ki67, neurofilament proteins (3). Strojnik et al. performed a CAM comparison study of rat brain and U87 human glioblastoma cell xenografts. It suggested that the CAM test is more suitable for invasiveness into the mesenchyme and vessel wall of small tumor nodules (37). Balassiano et al. demonstrated the anti-metastatic activity of perillyl alcohol on C6 glioma cells in a CAM model (2). Pen et al. demonstrated the tumor suppressive and vascular stabilization properties of Insulin-like growth factor binding protein 7 (IGFBP7) in U87MG and T98G glioblastoma cell lines transplanted onto CAM (27). Grodzik et al. studied the effect of carbon nanoparticles injected directly into tumors on the growth of human glioblastoma cells on CAM and showed that they reduced tumor volume and blood vessels in addition to lower expression of VEGF and FGF-2 in treated tumors compared to controls (12).

When the studies in the literature are examined, it is seen that the in vivo CAM model is a relatively inexpensive and easily applicable model, which allows Glioblastoma tumor formation, angiogenic and metastasis ability, detailed molecular and biochemical examination of tissues (34). However, the disadvantages of the CAM model are that it does not allow for long-term studies to evaluate the efficacy of anticancer agents and chickens are not mammals (30). This evidence requires further studies on the angiogenic and metastatic activity of glioblastoma in the CAM model, which is insufficient in the literature (30). In this study, we aimed to use the chicken embryo CAM model as an angiogenesis model for the development and treatment of malignant tumors of the central nervous system. Therefore, tumor tissue from Glioblastoma patients was transferred to CAM and invasiveness was observed on the 7th day. Changes in the area where the tumor was transplanted and in the surrounding tissues were examined by both histochemical and immunohistochemical methods. bFGF, PDGF, VEGF levels which is an angiogenic marker were evaluated comparatively between groups. There is no such study in the literature.

When compared histologically with control embryos, it was observed that there was an increase in the blood vessels in the CAM region where the tumor developed and an increase in the number of glial cell nuclei and inflammatory infiltration in the tumor tissue in the tumor transplanted embryos. Likewise, the increase in membrane thickness was evident in the samples taken from the CAM region distant from the tumor developed region. Significant hypercellularity, increase in blood vessels and inflammatory cells, and inflammatory infiltration in the connective tissue were observed in the connective tissue of the membrane.

When our immunohistochemical findings in terms of bFGF, PDGF, VEGF angiogenic factors were evaluated, it was noted that our angiogenic markers were expressed in the control group and that the expression of VEGF and PDGF was higher than bFGF in membrane epitel and connective tissue. It was determined that VEGF and bFGF immunostaining reacted more strongly than PDGF in the tumor transplanted groups, where the expression of all three markers increased in the tumor developed region. In the CAM region distant from the tumor developed region (from high to low), VEGF, bFGF and PDGF expressions were observed to increase compared to the control. As a result, bFGF, PDGF, VEGF staining, which are angiogenic factors, was observed to be more intense in tumor transplant groups than in the control groups. It was determined that this density was higher in the region where the tumor developed (Group 1) than in the region distant from tumor (Group 2).

CONCLUSION

According to this study, the tumor tissue (Glioblastoma) continues to develop after transplantation to the chicken chorioallantois membrane (CAM), and angiogenesis occurs with the increase in the expression of angiogenic factors. This change is seen not only in the area where the tumor developed, but also in the surrounding tissues. As a result, CAM could be a suitable model for CNS malignant tumor research. Although the protocol we created is a preliminary study on the use of therapeutic agents in cancer angiogenesis, it will also be a resource for other projects.

AUTHORSHIP CONTRIBUTION

Study conception and design: ASU, NU
Data collection: NU, SGG, ASU
Analysis and interpretation of results: NU, SGG, HYS
Draft manuscript preparation: NU
Critical revision of the article: SGG, ASU
All authors (NU, SGG, HYS, ASU) reviewed the results and approved the final version of the manuscript.

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