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Effects of Antibodies to EG-VEGF on Angiogenesis in the Chick Embryo Chorioallantoic Membrane

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Abstract. *Background: Endocrine gland-related vascular endothelial growth factor (EG-VEGF), is an angiogenic factor specifically targeting endothelial cells derived from endocrine tissues. The inhibition of the EG-VEGF/prokineticin receptor pathway could represent a selective antiangiogenic and anticancer strategy. Aim: to evaluate the impact of an antibody to EG-VEGF on the rapidly growing capillary plexus of the chick embryo chorioallantoic membrane (CAM). Materials and Methods: The in ovo CAM assay was performed for the humanized EG-VEGF antibody. Results: Hemorrhagic damage was induced in the capillaries, which led to early death of the embryos. Upon morphological staining, there was evidence of vascular disruption and extravasation of red blood cells in the chorion. Signs of vacuolization of the covering epithelium were also observed. Conclusion: Blocking endogenous EG-VEGF might represent a valuable approach of impairing or inhibiting angiogenesis in steroidogenic-derived embryonic tissues.*

Angiogenesis is the process of new blood vessel formation under normal conditions of wound healing and during reproductive functions. The mechanisms of angiogenesis have been extensively investigated because much data support their relationship with tumor progression. Moreover, antiangiogenic drugs have already been introduced into clinical practice (1). Even though tumor-associated endothelial cells have a unique proliferation and migration phenotype, hematological side-effects following the use of antiangiogenic drugs have been reported due to blocking of widely expressed growth factors or multiple tyrosine kinases (2). New hallmarks of the tumorigenic process have recently highlighted a microenvironment with tissue-restricted differentiation factors having a major influence on growth and morphological characteristics of tumor vessels, characterizing the so-called vascular address (3).

The concept of a tissue-specific angiogenic pathway was developed following the characterization of a novel human endothelial cell mitogen, the endocrine gland-derived vascular endothelial growth factor (EG-VEGF). Also called prokineticin-1 (PROK-1), this factor is not related to the VEGF family, but belongs to a class of structurally related peptides, which include the digestive enzyme co-lipase, a secreted cutaneous protein of the Yellow Belly toad (*Bombina variegata*) and a non-toxic compound from the venom of the Black Mamba snake (*Dendroaspis polylepis*) (4, 5). Prokineticins are involved in the regulation of vascular remodeling as well as in inflammatory responses. By targeting the prokineticin receptors (PROKR-1 and -2), EG-VEGF induces the proliferation, migration and survival of endothelial cells, under both physiological and pathological conditions. Moreover, PROK-1 overexpression has been correlated with several types of tumor: prostate, testicles, neuroblastoma, colon, and pancreas (6, 7).

The chorioallantoic membrane (CAM) has been reported to possess angiogenic and steroidogenic features (8). The present study aimed to evaluate the effects of a humanized antibody against EG-VEGF on the chick embryo CAM, which have not been previously reported.

Materials and Methods

Preparation of the chick embryo chorioallantoic membrane was performed according to the method previously described by Ribatti *et al*. (9). Briefly, on incubation day 3, 2 ml of albumin were removed and a window was created in the egg shell, covered with Parafilm and the egg specimens were re-incubated for the next four days. From incubation day 7, one group (n=5) was used as control and was treated with distilled water, whereas the other group (n=15, 5 specimens for each dilution) was treated with the anti EG-VEGF

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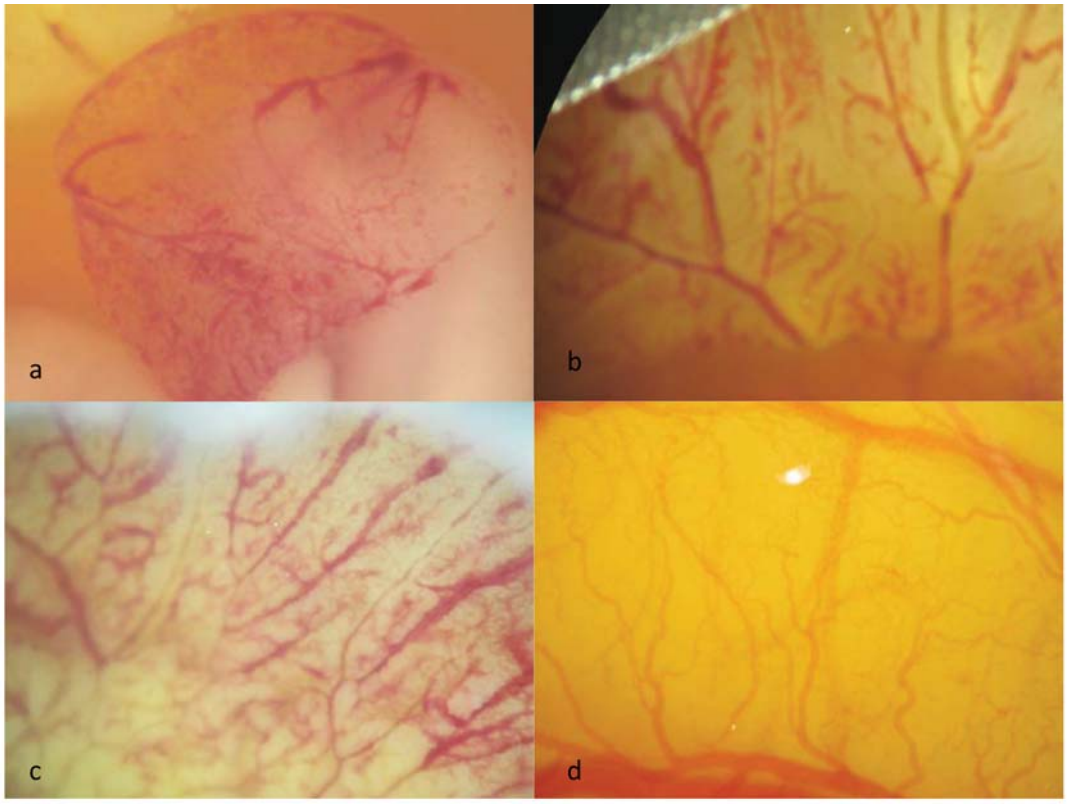

Figure 1. *Macroscopic view of chorioallantoic membranes (CAMs) on incubation day 8, one day after application of antibody against endocrine gland-related vascular endothelial growth factor (EG-VEGF). a: 1:50 dilution; b: 1:100 dilution, c: 1:150 dilution; d: distilled water; magnification ×18.*

antibody at different dilutions. The *in vivo* CAM assay was performed *in ovo* (9), by daily injecting different dilutions (1:50, 1:100, 1:150) of the EG-VEGF antibody (clone T-16, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in the embryo allantoic vesicle for two consecutive days. Treated and control CAM specimens were macroscopically monitored at 3, 6, 12 and 24 hours using a Zeiss Stemi DV4 SPOT stereomicroscope equipped with a Sony Cybershot camera.

Due to the low survival rate of the treated specimens and the rapid appearance of vascular changes, membrane specimens were collected on day 9 of incubation, fixed in formalin for 24 h and were then paraffin embedded. Five-micrometer serial sections were made from each specimen. Hematoxylin-eosin stain was used for the morphological study of the collected chick embryo CAMs. Additional sections were submitted to immunohistochemical staining for factor VIII (polyclonal rabbit antibody FVIII; Dako, Glostrup Denmark), Ki67 (ready to use antibody, clone MIB1; Dako), podoplanin (1:100 dilution, clone 18H5; Santa Cruz Biotechnology) using the LSAB+ -HRP (labeled streptavidin biotin – horseradish peroxidase) system and VEGFR3 (ready to use, polyclonal; LabVision, Neomarkers, Runcorn, Cheshire, UK), by using the EnVision detection system. The final reaction was visualized with 3,3'-diaminobenzidine (DAB), while modified Lille's hematoxylin was used for counterstain. The whole immunohistochemical procedure was performed in an automated fashion using the Dako Cytomation AutoStainer (Dako) and microphotographs were captured with a Nikon Eclipse E600. The Lucia G software system (Laboratory Imaging, Prague, CZ) was used for the processing of all images.

Results

Treated groups had a two-day limited survival rate after application of the anti EG-VEGF antibody. Just one day after application of antibody to EG-VEGF, the vascular network of treated CAM presented several macroscopic changes compared to the control group. All treated membranes showed hyperemic areas of the vascular plexus. Both the main vessels and the capillaries, had irregular shapes, with pronounced dilatation and severe stasis. In addition, extensive or spot-like hemorrhagic areas were observed between modified capillaries (Figure 1).

Concerning anti-EG-VEGF dilutions, differences were found between the treated groups. An intense hemorrhagic effect, restricted to the allantoic vesicle, was produced by the lowest (1:50) (Figure 1c) dilution of EG-VEGF antibody (Figure 1a), while the highest one (1:150) produced vascular changes extensive to the whole CAM surface, as in the embryos (images not shown).

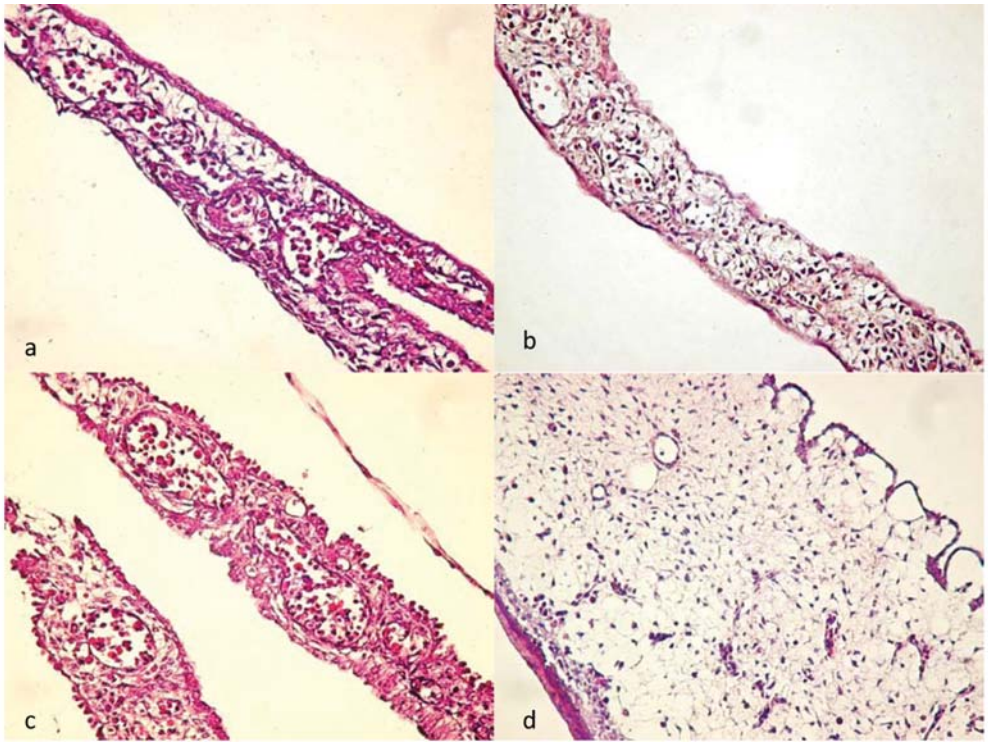

Figure 2. *Hematoxylin-eosin staining of chorioallantoic membranes (CAMs) on incubation day 9, two days after application of antibody against endocrine gland-related vascular endothelial growth factor (EG-VEGF). a: 1:50 dilution; b: 1:100 dilution, c: 1:150 dilution; d: distilled water; ×200 magnification.*

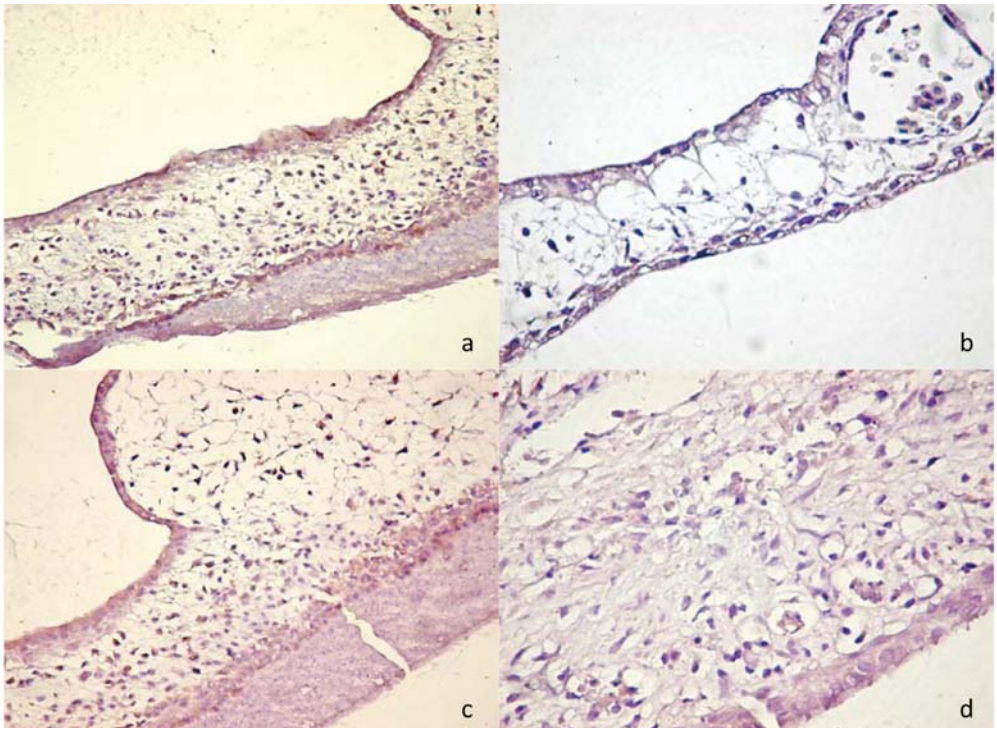

Figure 3. *Imunohistochemical (IHC) staining of chorioallantoic membranes (CAMs) on incubation day 9, two days after application of antibody against endocrine gland-related vascular endothelial growth factor (EG-VEGF) (1:150). a: factor VIII, magnification ×200; b: Ki67, magnification ×400, c: vascular endothelial growth factor receptor 3, magnification ×200; d: podoplanin, magnification ×400.*

Evaluation of microscopic specimens confirmed the macroscopic observations. On hematoxylin-eosin-stained CAM samples treated with antibody to EG-VEGF, we observed dilated medium and large vessels, and a high rate of extravasation of red blood cells in the mesencymal tissue of the CAM (Figures 2a, 2b and 2c). No significant differences in vessel density from treated and control groups (Figure 2d), were registered.

The effect of the antibody to EG-VEGF was not restricted to the vascular endothelium. Chorionic epithelial cells were modified by the action of the antibody to EG-VEGF, at a dilution of 1:150. Epithelial cells had many vacuoles into the cytoplasm and the adjacent stroma presented was denser using the 1:150 dilution of antibody (Figure 2c), compared with the normal mesenchyme from the control group (Figure 2d). Staining for the proliferation cell marker Ki67 was found to be slightly positive in some epithelial cells of the chorion exposed to the EG-VEGF antibody and absent from the endothelial cells from main vessels and capillaries (Figure 3b). Immunohistochemical reaction for the FVIII-related antigen revealed a discontinuous pattern on the endothelium in vessels from CAM, treated with 1:150 dilutions of antibody. These changes appeared to be related to (a) the pressure of the densified stroma on the blood vessels that tended to become organized as vascular bundle-like structures without lumen, or (b) to breakage of endothelial cell connections (Figure 3a).

By examination of the embryos, we observed massive hemorrhagic areas and blood stasis in several organs, especially in the brain and lungs.

The VEGFR3 expression was observed scattered in both normal chorion and the densified stromal tissues (Figure 3c). Staining for podoplanin was also slightly positive in the mesenchyme (Figure 3d).

Discussion

EG-VEGF is involved in several biological processes, including angiogenesis. EG-VEGF is overexpressed in steroidogenic glands and acts on endocrine gland endothelium (10).

The effects of EG-VEGF were extensively studied on human tissue specimens from pituitary gland (11) to human placenta (12). In birds, the CAM is similar to placental structures from humans and has the same function. Despite this, no data about the effects of EG-VEGF on chick embryo CAM have been previously reported and thus, to our knowledge, no data on an avian form of EG-VEGF exist. For this reason, and, given the fact that there is a 30% homology between the human and avian genomes, we chose the chick embryo CAM as an experimental model for testing effects of human anti the EG-VEGF antibodies *in vivo*.

Similar to VEGF, EG-VEGF is involved in the development of blood vessels in human endocrine tissues. It promotes endothelial cell proliferation, survival and chemotaxis. Previous findings of Brouillet *et al*. demonstrated the different behavior and particular molecular features of human placental macrovascular and microvascular endothelial cells treated with EG-VEGF *in vitro,* according to the differential expression of both PROKR1 and PROKR2 (12).

In this study, we demonstrated that the treatment with anti-EG-VEGF produced different effects on the main vessels and capillaries of the chick embryo CAM. After treatment with anti-EG-VEGF, the main vessels became dilated but preserved their structure, whereas the chick embryo CAM capillaries showed endothelial breakage with discontinuities through the intima and massive hemorrhage into the chorion. A common finding for both vessel types was the lack of Ki67-staining for proliferative endothelial cell activity, which is usually found in the normal chick embryo CAM vessels at this developmental stage, upon treatment with anti-EG-VEGF, whereas in control specimens the expression of Ki67 was restricted to nuclei of the basal cells from normal chorionic epithelium (data not shown).

No data are available regarding the effects of EG-VEGF on lymphangiogenesis. With the presence of positive reaction for both podoplanin and VEGFR3 reported in the present study, after the treatment with anti-EG-VEGF, we can speculate that anti-EG VEGF did not influence lymphatic vessel development. This could be indirect evidence of the lack of EG-VEGF involvement in the lymphangiogenic process.

Based on our observation concerning changes found in the chorioallantoic stroma and epithelium, we can hypothesize that in the avian embryo, an avian endogenous EG-VEGF exists and acts not only on endothelial cells, but also on other epithelial cell types and connective tissue components.

Chick embryo CAM is an avian steroidogenic structure (8) and this study showed that it is highly sensitive to blockade with EG-VEGF antibody in a dose-dependent manner. This suggests the presence of an endogenous avian type of EG-VEGF involved in the development of both, CAM and the embryonic vascular network. Moreover, changes caused by a human antibody to EG-VEGF of avian tissues suggest a high homology between human and avian endogenous EG-VEGF.

Conclusion

Chick embryo CAM represents a good tool for the study of EG-VEGF and their inhibitor effects *in vivo*. Further studies are required for the characterization of avian EG-VEGF, its receptors and the mechanisms of action in this type of experimental model. Characterization of an avian endogenous EG-VEGF and its corresponding inhibitors would improve the use of chick embryo choriallantoic membrane as a model for targeting the EG-VEGF pathways in normal and tumor angiogenesis but not lymphangiogenesis.

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