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The Chorioallantoic Membrane Assay Is a Promising *Ex Vivo* Model System for the Study of Vascular Anomalies

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Abstract. *The present feasibility study evaluated the chorioallantoic membrane (CAM) assay established in cancer and angiogenesis research as a tool for the study of vascular anomalies (VAs) in the head and neck area, since the lack of appropriate model systems poses a major obstacle in VA research. Materials and Methods: VA tissues from three patients, two with an arteriovenous and one with a lymphatic malformation, were analyzed and evaluated in the CAM assay. Results: The arteriovenous malformations induced a potent angiogenic reaction, resulting in new vessel growth and reperfusion by chicken embryo blood, which was comparable in extent with the positive vascular endothelial growth factor control. An angiogenic reaction, although less pronounced, was also observed in the single-tested lymphatic malformation. Conclusion: Our observations indicate the CAM assay to be a suitable model system for the study of VAs, as well as to show how treatment with pro- and antiangiogenic drugs affects VA growth patterns. The CAM assay has the potential to become a valuable tool for VA studies.*

Vascular anomalies (VAs) are a clinically and histopathologically heterogeneous group of benign lesions. They include hemangiomas, as well as vascular malformations such as lymphatic, venous and arteriovenous malformations (1). Patients with lymphatic and arteriovenous malformations in particular are frequently not only cosmetically-stigmatized, but also functionally-compromised (2). Depending on the anatomical site of the lesion, patients can develop problems with speech, swallowing and breathing, the latter often requiring a tracheostomy (3). Therapy options are limited and do not always achieve functionally- and cosmetically-satisfying results (4, 5). New therapies are thus needed in order to more effectively treat these lesions. In contrast to malignant tumors, however, there are only few experimental systems available for the study of VAs. In the present study, we evaluated the chorioallantoic membrane (CAM) assay, well-established as an *ex vivo* model in cancer and angiogenesis research (6, 7), regarding its suitability as a model system for VAs.

In the CAM assay, the extraembryonal chorioallantoic membrane is used to study angiogenesis. The CAM comprises a very dense capillary network, which serves as an optimal surface for the application of test substances, as well as tumor cells (6) and tumor xenografts (7). In contrast to other animal *in vivo* models, the chick embryo lacks a mature immune system, thus allowing the growth of xenografts without rejection (8), and the microscopic study and real-time quantification of tumor-induced angiogenesis (9, 10). Hence, the relatively simple and inexpensive CAM assay is also the method of choice for the analysis of angiogenic and anti-angiogenic effects in large-scale screening studies, and yields quantitative data within a few days (11). However, the use of the CAM assay for the analysis of VAs has not been explored so far.

Here, we describe the cultivation of biopsy specimens from two arteriovenous and one lymphatic malformation on the CAM.

Materials and Methods

Sample acquisition and preparation. Tissue material (Table I) was collected during regularly-scheduled surgical procedures at the Department of Otorhinolaryngology, University Hospital Marburg,

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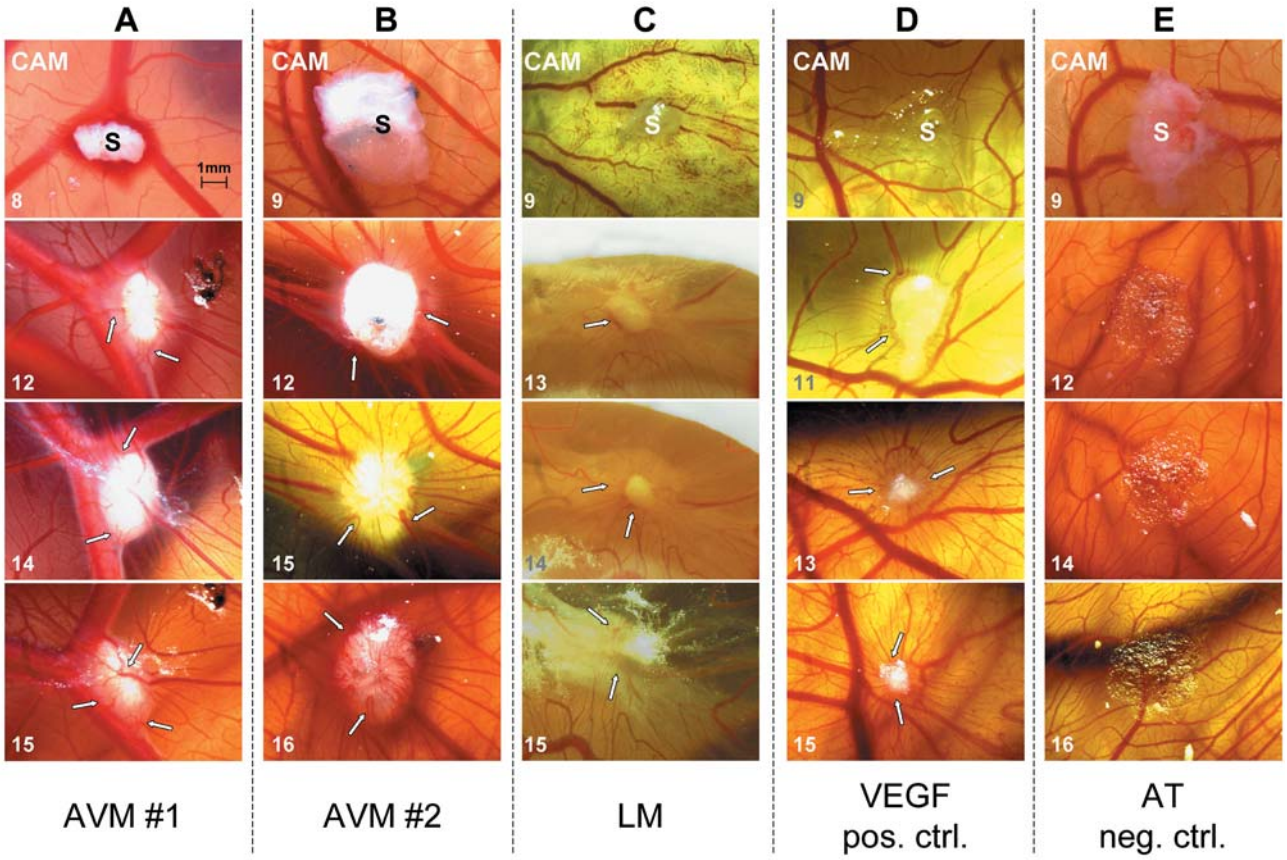

Figure 1. *Vascular anomalies (VAs) induce angiogenesis in the chorioallantoic membrane (CAM) assay. Representative micrographs of the three tested VA specimens are depicted in A-C. All arteriovenous malformation (AVM) tissues (A, B), as well as the lymphatic malformation (LM) specimen (C), induced angiogenesis (arrows). A sponge soaked with vascular endothelial growth factor (VEGF) served as a positive control (D), whereas adipose tissue (AT) (E) did not induce any angiogenic reaction. S, Specimen. Numbers in the lower left corner of each image correspond to the respective days of egg development. A scale bar is shown for size comparison.*

Germany. The study was approved by the local research Ethics Committee at the School of Medicine, Philipps University, Marburg, Germany (approval no.: 111/12). Prior to use, the biopsies were transferred into fresh pre-warmed Iscove's modified Dulbecco's medium (IMDM) (PAA, Coelbe, Germany), supplemented with 10% fetal calf serum (FCS). After 3 min, the biopsies were placed into 4 ml IMDM containing 1% penicillin/streptomycin/ amphotericin and 20% FCS and incubated for 1 h in a humidified atmosphere with 5% CO_2 at 37˚C for temperature equilibration prior to placement onto the CAM. VA tissue specimens also contained adjacent adipose tissue that was used as a negative control.

Preparation of CAM and inoculation of VA tissue samples. Upon delivery, fertilized specifically pathogen-free eggs were cleaned with an antiseptic solution (isopropanol 70%) and incubated at 37˚C and 60% relative humidity with gentle movement around the north-south axis of the egg. On day 4 to 5, a hole with a diameter of 3 mm each was drilled into both poles of the egg. From one of these holes, 5 ml albumin was removed and the hole was closed with tape in order to avoid further albumin efflux. At the broad pole, a window of 3 cm in diameter was opened and sealed with Parafilm®. The eggs were incubated at 37˚C and 60% relative humidity until egg development day (EDD) 8. On EDD 8, a 1-2 mm^3 piece of the respective biopsy specimen was placed onto the exposed surface of the CAM adjacent to an area with high blood vessel density. Subsequently, the window was closed again with Parafilm® and the eggs were incubated under the same conditions until EDD 16. Vascular endothelial growth factor (VEGF) was used as a positive control. In short, on EDD 9, a sterile gelatine sponge (Gelita-Tampon, Braun Melsungen AG, Melsungen, Germany) was cut into small cubes (approx. 1×2×2 mm). Each cube was soaked with 2 µl (1 µg/ml) of VEGF (Abcam plc, Cambridge, UK) to 2 ng VEGF/sponge. Subsequently the sponge was placed onto the CAM and vessel growth was monitored until EDD 15.

Analysis of angiogenesis. Starting from the day of tissue implantation, a daily analysis of the implants was carried out using a stereomicroscope (Carl Zeiss Microscopy GmbH, Göttingen, Germany) connected to a digital camera (Moticam 2000; Motic China Group, Hong Kong, China). Each image was captured using the Motic Images Plus 2.0 ML software at 1600×800 pixel resolution.

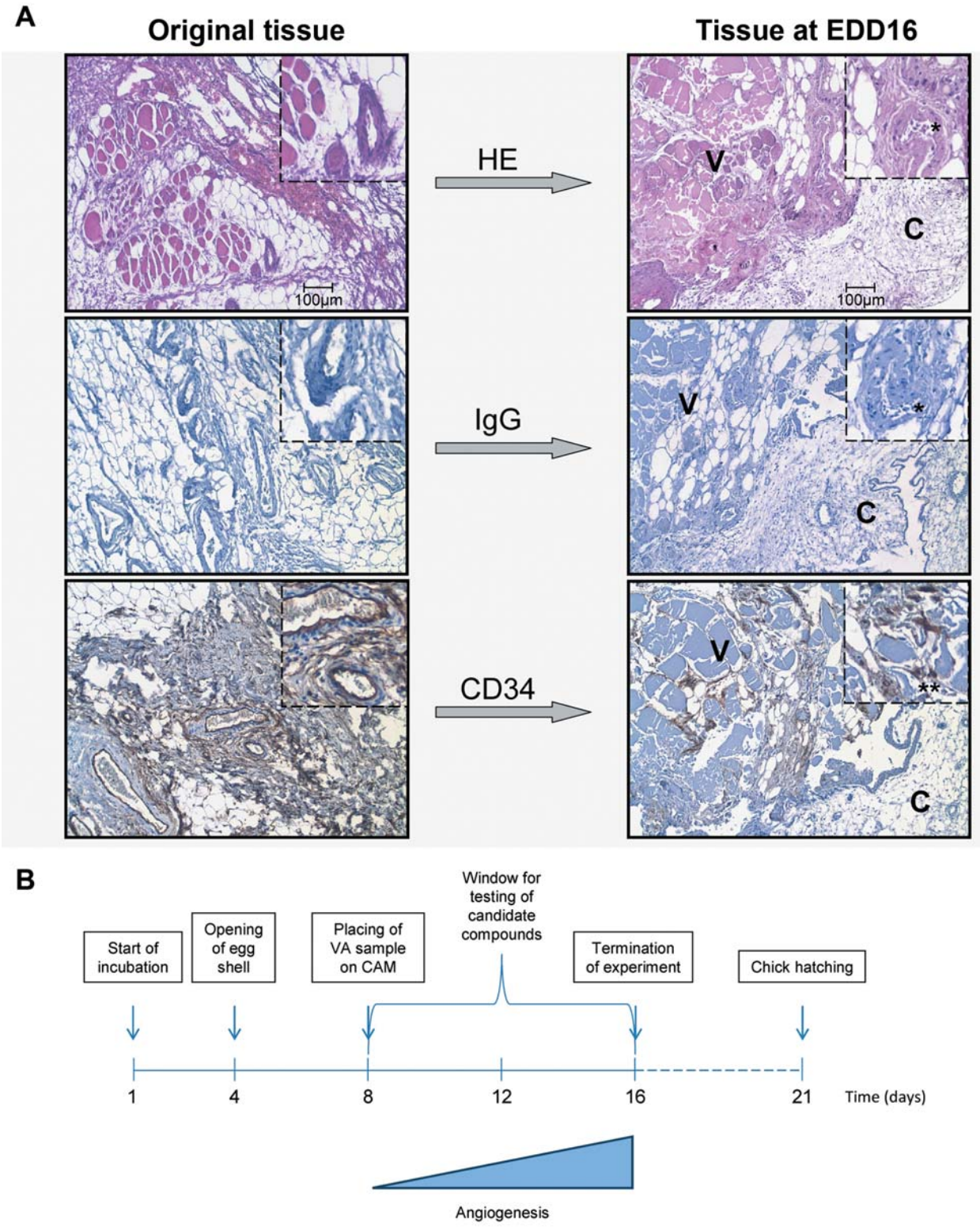

Figure 2. *Arteriovenous malformation (AVM) tissue, derived from the chorioallantoic membrane (CAM) retains similarity to the tissue of origin. A: The CAM-derived AVM tissue (AVM #2) exhibits regions of vessel reperfusion visible by the presence of chicken-derived nucleated erythrocytes (*). Lower panel: Immunohistochemical staining shows abundant cluster of differentiation 34 (CD34)-positive endothelial cells (**) in the region of the AVM specimen (V), whereas the CAM (C) exhibits no positive labeling. B: Time scheme, depicting the procedure and highlighting the time window of vessel growth which can be exploited for testing pharmaceutical candidate compounds. A scale bar is shown for size comparison.*

Table I. *Clinical information of the three tested vascular anomaly specimens.*

Label	Type	Location	Side	Gender	Age (years)
AVM #1	Arteriovenous malformation	Face and nose	Right	Male	33
AVM #2	Arteriovenous malformation	Face	Left	Female	37
LM	Lymphatic malformation	Face and neck	Right	Male	15

AVM: Arteriovenous malformation; LM: lymphatic malformation.

Histopathology. Tissue samples were formalin-fixed and paraffin-embedded (FFPE). Three micrometer-thick tissue sections of the original arteriovenous malformation (AVM) tissue (AVM #2), as well as the respective tissue from the CAM assay, were cut with a microtome and stained with hemalaun/eosin/erythrosin (Merck, Darmstadt, Germany) according to a standard protocol. For immunohistochemical analysis, a mouse monoclonal antibody directed against the endothelial cell marker cluster of differentiation 34 (CD34) (QBEnd-10, M7165; Dako, Glostrup, Denmark) was used at a dilution of 1:50 according to the manufacturer's protocol. As a negative control, non-specific mouse IgG1 (Negative Control, X0931; Dako) was used at a dilution of 1:50. Staining results were evaluated by light microscopy (AX70; Olympus, Hamburg, Germany).

Results

VA specimens, pre-treated as described and cut into pieces, were placed onto the CAM of different eggs at EDD 8. To account for variations, at least 20 eggs were used and subsequently monitored for vessel growth. When detectable, angiogenesis was typically found in at least 40% of the eggs. Representative images taken at different time points are shown in Figure 1. Both AVMs induced an angiogenic response that was particularly prominent during EDD 12 to 16 (Figure 1A and B). Interestingly, the tested lymphatic malformation also induced vascular growth, although not as pronounced as that seen in the two AVM tissues (Figure 1C). The positive control (VEGF-soaked sponge) provoked angiogenesis to a similar extent to that observed in the case of the two AVMs (Figure 1D) whereas adipose tissue used as a negative control did not elicit any vascular reaction (Figure 1E). As depicted in Figure 2A, the tested AVM tissue was reperfused with chicken embryo blood (asterisks indicating erythrocytes with a nucleus), thus confirming the re-vascularization of the specimen.

We also compared blood vessel staining of the primary AVM tissue sample versus the CAM xenograft from EDD 16 (Figure 2A, lower panel). The xenografted tissue presented CD34-positive endothelial cell structures (two asterisks) comparable to the structures found in the original surgical sample. In contrast, the adjacent CAM did not exhibit CD34 immunopositivity (Figure 2A).

Discussion

Precisely 100 years ago, Murphy published the first article describing the cultivation of foreign tissues on chicken embryo CAM (12). The CAM assay has been extensively used since then in cancer and angiogenesis research (13). These CAM assay applications, however, rely on transformed tissues/cells that are characterized by autonomous, unrestricted growth. Less attention was paid to studies of non-transformed benign tissues, probably because these tissues are expected to be less prone to growing in a CAM assay. VAs are rare diseases and are benign in nature (14). They are characterized by abnormal vessel growth and architecture and are of pre-eminent clinical relevance (15, 16). In the present feasibility study, we demonstrated that VAs grow reliably in the CAM assay and have the propensity to induce angiogenesis and re-vascularization. These observations indicate the suitability of the CAM assay as a platform for pharmaceutical studies of VA tissues, being able to directly address the tissue response to known pro- and anti-angiogenic therapies, as well as new experimental candidate compounds (Figure 2B). Another major advantage of the CAM assay is that only small amounts of tissue samples are typically required for *ex vivo* testing, and angiogenesis can be more easily and quantitatively monitored in real time. This allows for response evaluations of specific VA tissues, requiring only a small biopsy of the vascular lesion. Given its relatively short duration, one could envisage pre-therapeutical tests performed in the CAM assay to identify effective medications prior to treatment of the patient from whom the biopsy was derived. The CAM assay, in the context of assessing the pharmacological response of VAs, has a high potential for contributing to personalized medicine by functioning as a relatively easy xenopatient model. However, although the CAM assay is highly promising for such investigations, technical difficulties require careful interpretation of the results. For example, as reported by Staton *et al.*, due to the vascularization of the CAM itself, it can be difficult to distinguish new capillaries from pre-existing blood vessels (11). Similarly, Auerbach *et al.* report that the CAM is a very suitable *in vivo* model

system but results need to be interpreted carefully since the involved cellular structures are derived from a species (chicken) usually differing from the tested tissues (17). In addition, more than one egg per specimen is required to obtain reliable results. A future task will also be the development of standard-operating procedures for the exact handling of VA tissue specimens. Subsequent studies will further explore the reliability of the CAM assay for the *ex vivo* testing of VAs.

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