Pathol. Oncol. Res. (2011) 17:309–313
DOI 10.1007/s12253-010-9318-y

RESEARCH

Definition of the "Drug-Angiogenic-Activity-Index" that Allows the Quantification of the Positive and Negative Angiogenic Active Drugs: A Study Based on the Chorioallantoic Membrane Model

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Received: 20 April 2010 / Accepted: 6 October 2010 / Published online: 8 January 2011

Abstract Since the introduction of the angiogenic therapy by Folkman et al. in the 1970'ies many antiangiogenic drugs were identified. Only few of them are still now in clinical use. Also the Vascular Endothelial Growth Factor (VEGF), the cytokine with the highest angiogenic activity, has been identified. Its antagonist, Bevacizumab, is produced and admitted for the angiogenic therapy in first line for metastatic colorectal cancer. When we look at preclinical studies, they fail of in vivo models that define the "Drug-AngiogenicActivity-Index" of angiogenic or antiangiogenic drugs. This work proposes a possible standardized procedure to define the "Drug Angiogenic Activity Index" by counting the vascular intersections (VIS) on the Chorioallantoic Membrane after drug application. The equation was defined as follows: {ΔVIS[Drug]−ΔVIS[Control]} / Δ VIS[Control]. For VEGF a Drug-Angiogenic-Activity-Index of 0.92 was found and for Bevacizumab a −1. This means almost that double of the naturally angiogenic activity was achieved by VEGF on the Chorioallantoic membrane. A complete blocking of naturally angiogenic activity was observed after Bevacizumabs application. Establishing the "Drug-Angiogenic-Activity-Index" in the preclinical phase will give us an impact of effectivness for the new constructed antiangiogenic drugs like the impact of effectiveness in the cortisone family.

Keywords Angiogenesis · Chorioallantoic membrane · Drug angiogenic index · Antiangiogenic therapy

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Introduction

More and more drugs are being designed for antiangiogenic therapy in cancer research since their discovery by Judah Folkman. [1, 2]. "Drug-Angiogenic-Activity-Index" (DAAI) should be established in vitro and in vivo models in preclinical studies. This is necessary for evaluating the clinical effect into patients. Also the side effects of the known antiangiogenic agents could be estimated, just like the therapy using the cortisone drug family [3]. Actually, Bevacizumab (Avastin®), a VEGF-antibody, is admitted in the therapy for metastatic colorectal cancer [4, 5]. Many other antiangiogenic agents for other carcinomas will follow in the near future.

The angiogenic activity of VEGF and the antiangiogenic activity of Bevacizumab were studied as a baseline for the other angiogenic molecules for defying the Drug-Angiogenic-Activity-Index (DAAI).

For this purpose the Chorioallantoic Membrane (CAM) assay was used. It is a relatively simple and inexpensive in vivo model, suitable for large-scale screening for angiogenesis activity of drugs [6].

However an overview of the literature shows no comparable results between different study groups. It lacks of a standardized treatment. Several techniques are described to get to CAM. When and where should be the eggs shells opened? In which position should be the eggs hatched?

In principle, two ways of opening techniques are described. First, the eggs are placed in horizontal position and are opened after two days of breeding on the side of equator [7]. In the second method, the eggs are hatching in upside position and are opened on the blunt side on day 9 or 10 of breeding [8].

The way of drug application defers also. Just dropping drugs on the CAM makes no sense, because first you observe an uncontrolled distribution of the drug onto the CAM's surface and second recognizing the area for

investigation after drug application on the developing CAM is almost impossible.

That's why a vehicle is needed to mark and fix the drug by a defined area on the CAM. Three main vehicles are described in the literature. The test substance is prepared either in slow-release polymer pellets, absorbed by gelatine sponges [9], or air-dried onto plastic discs [7]. Moreover a silicon ring as a vehicle is described by Kunzi-Rapp for investigation of the apoptosis and seeding in prostate cancer cells, without quantification of angiogenesis [10].

Quantification of angiogenesis is mostly performed after histological stains.

After selection of the above given Methods a standardized protocol is provided.

Material and Methods

Eggs and Preparation

Fertilized chicken eggs (White Leghorn, 50–54 g, Charles River Wiga, Sulzfeld, Germany) were disinfected with a sterile compress saturated with 70% of ethanol and incubated for 9 days, in an upright position with the blunt pole on top (Fig. 1a), into chambers with 37°C temperature, 80% humidity and normoxia (20.5% O_2) After nine days the eggs were preparated as it follows: at the beginning a 2 mm hole was milled carefully into the egg shell on the zenith of the blunt pole using a graving instrument (Proxon Micromat System®, Niersbach, Germany). The dust generated during milling was removed by a humidified sterile compress. The outer shell membrane was not ruptured. Then anatomical forceps were used to perforate of the outer shell membrane and open the air cell. Subsequently the egg shell was broken according the topography of the air cell. This way a circled window of 2–3 cm could be opened up (Fig. 1b–c). Using a stereomicroscope, the inner shell membrane was humidified with 0.5 ml of Ringers` solution. This procedure allows removing the inner shell membrane with micro tweezers without destroying the epithelial layer and the vascular architecture of the CAM (Fig. 1d).

Then a sterile ring (Diameter: 5 mm; height: 3 mm) was cut out of a plastic straw (polypropylene) and it was placed in the center of the CAM (Fig. 1e). The ring works as a chamber for further experiment. The window was closed with a corresponding size of glass and tape. The eggs were put back in the incubator for one day. Waiting one day for further manipulation means that the eggs can recover and the ring will be embedded on the CAM in a way that the applied drug won't run out of the ring. To demonstrate that Methylenblue was dropped in the ring (Fig. 1f–h) and we could see that it stays in the Ring without causing diffuse distribution.

After one day of recovery, on day 10 of breeding, the eggs were taken for further studying. Before inoculation of either Ringers solution, VEGF or Avastin® into the chamber, a photo of the CAM area was made with 2,5× magnification (Camera, Canon®, Japan and Zeiss, Stemistereomicrocope®, Germany) (Fig. i–j). The magnification of the CAM allows to view and count vessels with a diameter above 10 μm.

Drugs and Application

Ringers solution 10 μl, as control-group (n=8, Cont.-Group), Vascular Endothelial Growth Factor 165 (n=8; Concentrations: 500 ng à 10 μl) as positive group ($VEGF_{500}$-Group) and recombinant human VEGF antibody, Bavacizumab (n=7; 350 μg à 10 μl Avastin®) negative group were applied in the ring via pipette on 10th breeding day.

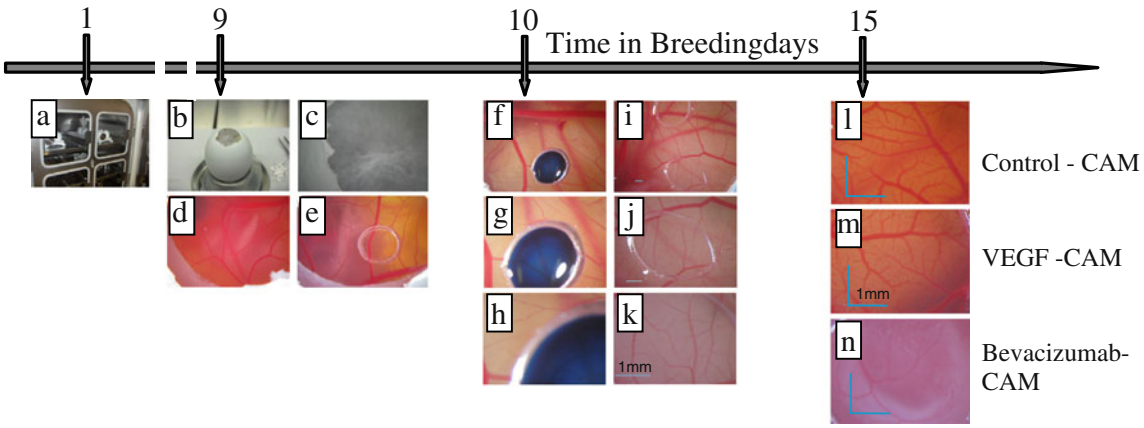

Fig. 1 Chorioallantoic Model (CAM) Assay for investigating Drug-Angiogenic-Activity-Index Work flow in breeding days (BD) (**a**). During the first 9 BD the eggs were incubated in an upright position and were opened on the blunt pole (**b**). To get the chorioallantoic membrane out, the inner shell membrane was removed (**c**–**d**). Then a ring (Diameter: 5 mm; height: 3 mm) was cut out of a straw and it was placed in the center of the CAM. The ring works as a chamber for further experiment. Methlyenblue was dropped in to the ring for demonstrate that the drugs stay in the ring (**f**–**g**). On 10th BD either VEGF or Bavacizumab was applicated. The quantification of the angiogenic activity was performed on 10th (**i**–**k**) and 15th BD (**l**–**m**)

Quantification of Angiogenesis

Counting the vascular density, with the method of counting Vascularintersectiones per mm^2, gives information about the angiogenesis activity on the CAM [8]. For this reason, photos with a magnification of 2.5× on the Stereomicroscope were taken from inner ring of the CAM, just before the application of drugs and after 5 days of incubation (Fig. k–n). To verify the original size of the CAM, a millimeter paper was photographed using the same scale. Then a 1 mm^2 square was applied to the photo which was taken of the inner ring CAM (Fig. 2) using Adobe-Photoshop®. After that, all the vessels were counted either horizontally or vertically, crossed on the vertical or horizontal line (Fig. 2). The multiplication of both numbers vertically and horizontally was equal to the vascularintersectiones per mm^2 (VIS/mm^2) on the CAM.

The "Drug-Angiogenic-Activity-Index" was defined by counting the VIS/mm^2 on 10th and 15th BD at each group as by the following equation:

$$\frac{\{VIS^{15thBD}[Drug] - VIS^{10thBD}[Drug]\} - \{VIS^{15thBD}[Control - Group] - VIS^{10thBD}[Control - Group]\}}{VIS^{15thBD}[Control - Group] - VIS^{10thBD}[Control - Group]}$$

Statistics

Statistical analyses were performed using the Mann-Whitney-Wilcoxon independent-samples Test and the statistic software SPSS® (SPSS version 18.0 for Windows, SPSS Inc.). P values <0.05 were considered statistically significant.

Results

The developing CAM (control-group) increases its vascular density from 8±1 VIS/mm^2 on 10th BD to 20±2 VIS/mm^2 on 15th BD (Figs. 3 and 4).

Recombinant human VEGF^{165} increases the vascular density from 10th BD 8±2 VIS/mm^2 to 31±6 VIS/mm^2 at the 15th BD on the CAM significantly ($p<0.05$).

$$\frac{\{VIS^{15thBD}[VEGF] - VIS^{10thBD}[VEGF]\} - \{VIS^{15thBD}[Control] - VIS^{10thBD}[Control]\}}{\{VIS^{15thBD}[Control] - VIS^{10thBD}[Control]\}} = \frac{(31-8)-(20-8)}{(20-8)} = 0.92$$

For VEGF a Drug-Angiogenic-Activity-Index of 0.92 was calculated.

Bevacizumab (Avastin®) blocked the increase of the vascular density on the CAM significantly. On 10th BD the

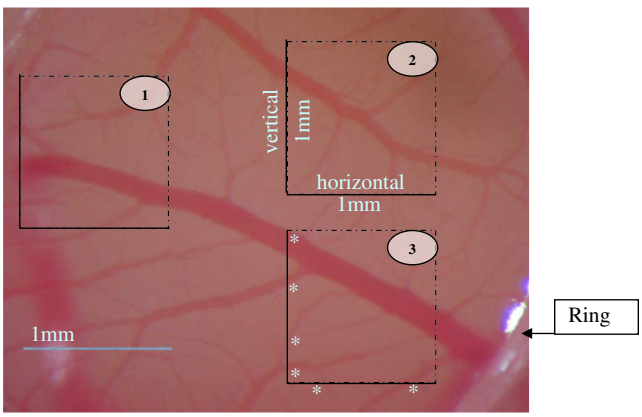

Fig 2 Quantification of the Angiogenic Activitiy: This photo was made with 2.5× magnification on the Streomicroscope at CAM on the 10th BD. The original size is given with blue line of 1 mm.Vertically and horizontally lines of 1 mm were applied. Each of the vascular intersectiones with this lines (*) were counted and multiplicated. This resulted in the vascularintersectiones of an area of 1 mm^2. This was repeated three times, randomly (1–3). And the middle of this calculation produced the vascularintersections/mm^2 of one sample

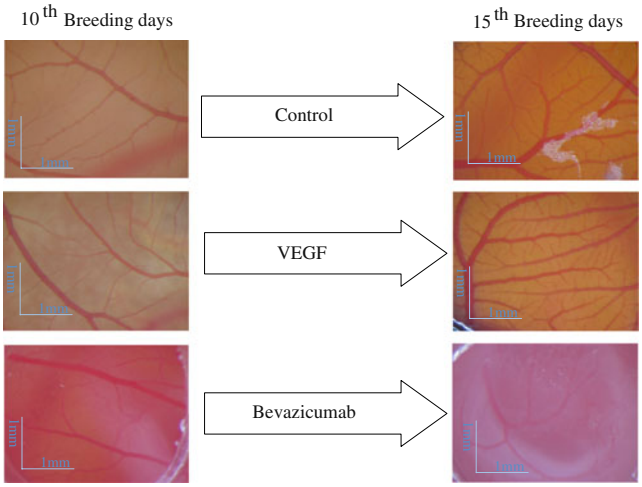

Fig. 3 Examples for the Chorioallantoic membrane before (on 10th BD) and after drug application (on 15th BD)

vascular density was 5±1 VIS/mm^2 and on 15th BD the vascular density did not increase (5±1 VIS/mm^2).

$$\frac{\left\{\mathrm{VIS}^{15\mathrm{thBD}}[\mathrm{Bevacizumab}]-\mathrm{VIS}^{10\mathrm{thBD}}[\mathrm{Bevacizumab}]\right\}-\left\{\mathrm{VIS}^{15\mathrm{thBD}}[\mathrm{Control}]-\mathrm{VIS}^{10\mathrm{thBD}}[\mathrm{Control}]\right\}}{\left\{\mathrm{VIS}^{15\mathrm{thBD}}[\mathrm{Control}]-\mathrm{VIS}^{10\mathrm{thBD}}[\mathrm{Control}]\right\}}=\frac{(5-5)-(20-8)}{(20-8)}=-1$$

For Bevacizumab a Drug-Angiogenic-Activity-Index of −1 was calculated.

Discussion

This paper describes a standardized procedure for quantification of "Drug-Angiogenic- Activity-Index". Upright breeding of the egg has two advantages. First, the eggs don´t occupy much place in the incubator. Second, the air cell has been encountered constantly on the blunt pool of the egg. The opening technique from the blunt pool according to the topography of the air cell allows the observation of a 3 cm in diameter window and also allows putting the ring on the CAM easily. Using the ring as a tracer for drug application has an importand advantage: Only an exactly defined area of the CAM is influenced by the drug.

An angiogenic activity increase was detected naturally on the CAM from 8 to 20 VIS/mm^2 (Figs. 3 and 4) from 10th to 15th BD. This was considered as the denominator of the equation for the "Drug-Angiogenic-Activity-Index". Looking at the extreme values gives an idea of this equation (Fig. 5). A DAAI of 1 means that twice of the naturally increasing angiogenic activity of the CAM could be achieved. DAAI of −1 means that the naturally increasing angiogenic activity is completely blocked at the stage of 10th BD of the CAM. A DAAI of <−1 means a decrease and destruction of the vessels. After application of VEGF almost twice (DAAI 0.92) of the naturally angiogenic activity was detected. For Bevacizumab (Avastin®) a DAAI of −1 was calculated, which means a completely block of the naturally increasing angiogenic activity.

Defining a Drug-Angiogenic-Activity-Index in the preclinical phase gives an idea of the potential desired and undesired clinical effects of the angiogenic active drugs. Further investigation will be concentrated for finding the DAAI of other antiangiogenic drugs like Cetuximab, Interferon α, Interferon γ or new ones so that we can have data about the expected effects in the clinical use.

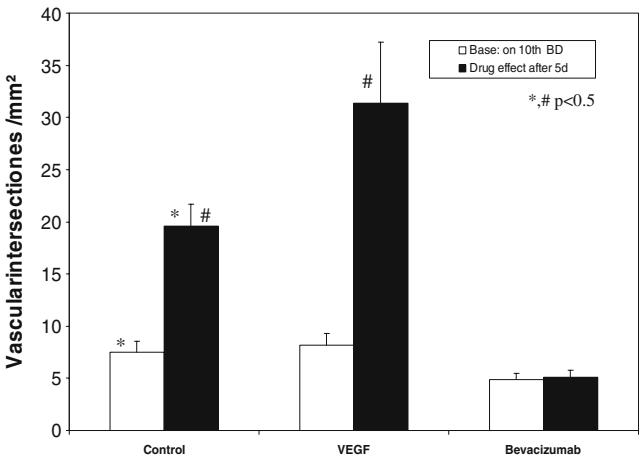

Fig. 4 Vascular intersectiones per mm^2 before, at 10th BD and after drug application at 15th BD were demonstrated. A naturally and significantly ($p<0.5$) increasing of the VIS/mm^2 was seen after 5 days of incubation from 8 to 20 VIS/mm^2 (*). An additionally significant ($p<0.05$) increasing of the VIS was demonstrated after application of VEGF to 31 VIS/mm^2 (#). The angiogenic activity was blocked after Bevacizumab application (5 VIS/mm^2) and stayed at the stage of the 10th BD

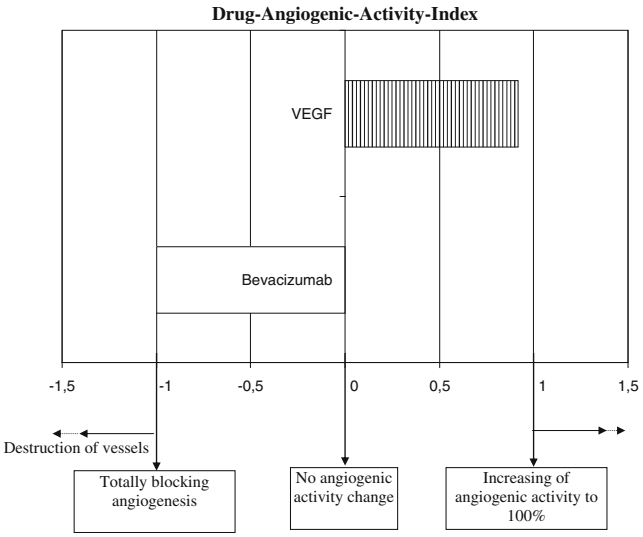

Fig. 5 Drug-Angiogenic-Activity-Index "0" means no angiogenic activity change on the chorioallantoic membrane occurred after 5 days of observing time. The naturally increasing of angiogenesis on the CAM is unaffected. Application of 500 ng of VEGF produced a DAAI of 0.92 on the chorioallantoic membrane. That is closed to 100% of increase of angiogenic activity of the CAM. Application of Bevacizumab totally blocks naturally angiogenic activity

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