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Note

A chorioallantoic membrane model for the determination of anti-angiogenic effects of imatinib

Aybike Özcetin [a], Achim Aigner [b], Udo Bakowsky [a,*]

[a] Department of Pharmaceutical Technology and Biopharmaceutics, University of Marburg, Marburg, Germany
[b] Rudolf-Boehm-Institute for Pharmacology and Toxicology, University of Leipzig, Leipzig, Germany

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ABSTRACT

Tumor angiogenesis is of major importance in the growth and metastasis of solid tumors, and the development of anti-angiogenic treatment strategies is thus a relevant option in oncology. The chorioallantoic membrane (CAM) model is a rapid and simple alternative to *in vivo* studies for the evaluation of anti-angiogenic compounds, thus allowing to reduce animal experiments and, upon establishment of robust and reproducible procedures, to more efficiently and objectively assess the anti-angiogenic efficacy of a given drug.

In this paper, we compare two different methods for tumor establishment on a CAM model: (i) a Murine Urothelial Carcinoma (MB49) cell suspension mixed with Matrigel and (ii) an MB49 cell suspension absorbed in Gelfoam gelatin sponges. Based on the applicability of both methods for implant formation, we identify Gelfoam gelatin sponges as superior due to better attachment of the tumors on the membrane surface. For the precise quantitation of tumor xenograft growth and angiogenesis, we furthermore establish in this paper the electronic capturing of the xenografts and the computer-based analysis of the microscopic CAM images in order to determine the number of intersecting vessels and to measure vessel diameters.

Beyond its direct effect on tumor cells by inhibiting the tyrosine kinase domain of the *abl* gene, imatinib has been reported to reduce the Bcr-Abl-mediated secretion of the angiogenesis factor VEGF and hence to interfere with angiogenesis. To test our CAM model for its ability to monitor anti-angiogenic effects, Gelfoam gelatin sponge-based tumor implants were treated by topical application of imatinib at various concentrations. Besides anti-tumor effects, we observed an inhibition of angiogenesis as determined by the number or total diameter of intersecting vessels. We also demonstrate that the calculation of the "blood vessel index" (vessel total diameter/tumor circumference) in our model allows to assess anti-angiogenic effects of imatinib independently of tumor growth inhibition. We conclude that our CAM assay and computer-based analysis represent a useful in vitro technique for the rapid assessment of anti-angiogenic effects of various agents.

1. Introduction

It is well established that solid tumors rely on the formation of new blood vessels as an essential prerequisite for their supply with oxygen and nutrients. Under normal conditions, angiogenesis, i.e. the formation of new blood vessels from existing ones, is very restricted in adults, only occurring during the ovarian cycle, wound healing, and some other (patho) physiological processes that involve tissue growth or repair [1]. However, most solid tumors > 2–3 mm^3 cannot survive without blood vessel supply, thus establishing the necessity of angiogenesis for tumor progression and, at a later stage, for metastasis [2]. Consequently, anti-angiogenesis is regarded as a promising strategy in cancer therapy. Angiogenesis relies on the proliferation and migration of endothelial cells, which is linked to the expression of growth factors by tumor cells. Several growth factors have been shown to be involved in tumor angiogenesis, including basic fibroblast growth factor (bFGF; FGF2), vascular epithelial growth factor (VEGF), platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-β), angiopoietin-1, integrins, and the platelet endothelial cell adhesion molecule (PECAM-1) [3]. Inhibitors of any of those molecules may thus exert anti-angiogenic effects that are relevant in tumor therapy. Their assessment, however, requires the precise analysis of these effects in suitable experimental models.

The chorioallantoic membrane (CAM) model is a rapid and simple alternative to *in vivo* studies for the evaluation of anti-angio-

* Corresponding author. Department of Pharmaceutical Technology and Biopharmaceutics, University of Marburg, Ketzerbach 63, D-35037 Marburg, Germany. Tel.: +49 6421 282 5881; fax: +49 6421 282 7016.
E-mail address: ubakowsky@aol.com (U. Bakowsky).

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genic compounds. It thus allows to reduce animal experiments and to more efficiently and objectively assess the therapeutic (here anti-angiogenic) action of a given drug. The CAM is an extraembryonic membrane of the chicken egg, comprising a very dense capillary network and mediating the exchange of gas and nutrients to the embryo. Since the immunocompetence of the chicken is not fully developed during the embryonic stage, xenografts can be established without rejection [4], prior to monitoring new vessels that arise from the host and grow toward the tumor [5]. Notably, the tumor can transfer growth signals over a distance of up to 5 mm, and if a microvessel fails to penetrate the tumor until the third day of tumor implantation, the tumor remains pale. Tumor-induced microvessels are tiny and have short half-lives, and if the stimulus is turned off, they regress quickly [6]. Therefore, the anti-angiogenic effects of an inhibitor can be accurately determined in a CAM assay by microvessel counting. However, the application of oncologically relevant test compounds onto the CAM model may also in parallel exert tumor cell inhibition, leading to macroscopic anti-tumor effects in the xenografts. While this allows the analysis of cytostatic effects as well as the assessment of efficacies of photodynamic or radiotherapeutical applications in CAM assays, it may obscure the precise quantitation of anti-angiogenic effects, thus requiring a more sophisticated analysis of vessel density data.

Imatinib (Glivec, STI 571) is a phenylaminopyrimidine analog that binds to the ATP-binding site of the tyrosine kinase domain in the *abl* (Abelson murine leukemia) gene. In diseases such as chronic myelogenous leukemia, the Philadelphia chromosome leads to a fusion protein of *abl* with the *bcr* (breakpoint cluster region) gene which causes a constitutively active tyrosine kinase, and imatinib exerts profound therapeutic benefits by inhibiting the tyrosine kinase activity [7]. Notably, it was also reported that imatinib reduces the secretion of VEGF, which is mediated by the Bcr-Abl complex, and thus may be linked to anti-angiogenesis [8]. In this context, it has also been shown that imatinib exerts anti-angiogenic effects in connection with PDGF/PDGFR signaling pathway, when overexpression of PDGFR during vasculoproliferative diseases is observed [9]. This prompted us to test our CAM assay regarding anti-angiogenesis effects of imatinib.

In this paper, we (i) identify an optimal method for tumor cell implantation onto the CAM, (ii) establish the computer-based analysis of angiogenesis, (iii) demonstrate that this model allows the rapid and precise quantification of anti-angiogenic effects independent of anti-tumor properties, and (iv) analyze imatinib with regard to its anti-angiogenic potential.

2. Materials and methods

2.1. Materials

Tumor implants were prepared using the murine urothelial carcinoma cell line MB49 (ATCC, Manassas, VA) with matrigel (BD Matrigel™ Basalmembranmatrix, BD Biosciences, Bedford, MA) or with a gelatine sponge (Gelfoam, Pfizer, Pharmacia & Upjohn, Kalamazoo, MI). Imatinib was purchased from LC Laboratories (Woburn, MA). Fertilized VALO-SPF eggs were obtained from Lohmann Tierzucht (Cuxhaven, Germany). Cells were cultivated at 37 °C in a humidified atmosphere with 5% CO_2 in Iscove's Modified Dulbecco's Media (IMDM) medium (PAA, Coelbe, Germany), containing 10% fetal calf serum (FCS) (PAA) and 1:100 (v/v) penicillin/streptomycin (PAA).

2.2. Experimental methods

2.2.1. Preparation of CAM

Upon delivery, fertilized specific pathogen-free eggs were cleaned with an antiseptic solution and incubated at 37° C and 60% relative humidity. On day 5, two holes with a diameter of 3 mm each were drilled into both poles of the egg. From one of these holes, 6–7 ml albumin was removed, and the holes were closed with tape to avoid further albumin efflux. On the broad pole, a window with 2.5 cm in diameter was opened and sealed with Parafilm, and until day 18, the eggs were incubated at 37 °C and 60% relative humidity.

2.2.2. Preparation of Gelfoam tumor implants

On days 8–10, a ~2 mm^3 Gelfoam piece was cut under sterile conditions and wetted with 10 µl MB49 cell suspension (3×10^6 cells/ml in serum-free medium). Thus, each sponge contained approximately 30,000 cells and was placed on the CAM at a position where the blood vessel density was high [5].

2.2.3. Preparation of Matrigel tumor implants

Matrigel tumor implants were prepared 1 day before implantation. Matrigel stored at −20 °C was warmed up to 4 °C and homogenized by shaking. 5×10^6 cells in 20 µl cell medium were mixed with 10 µl matrigel solution and incubated overnight in 24-well plates. The next day, the matrigel-cell mixture was removed from the wells and placed on the CAM at a position where the blood vessel density was high [5].

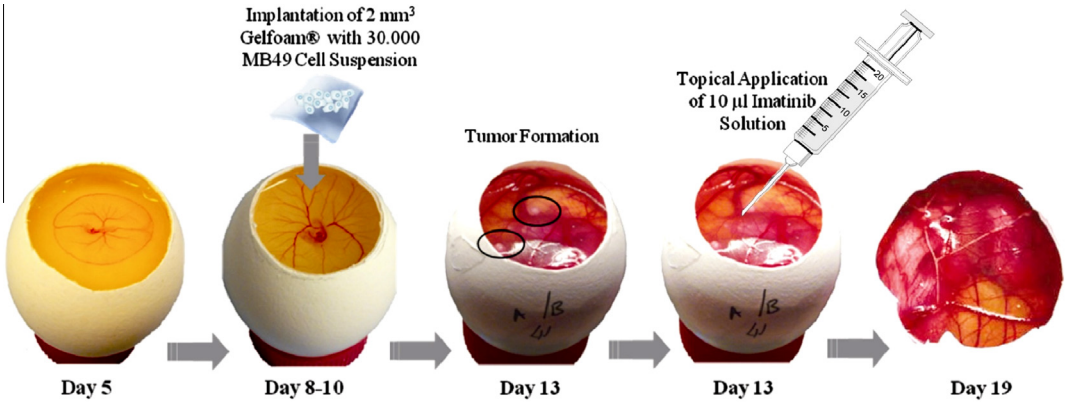

Fig. 1. Photographic overview of the execution of the Gelfoam-based CAM assay. On day 5, the embryo and the beating heart could be observed. Between days 8 and 10, a 2 mm^3 Gelfoam piece containing an MB49 cell suspension with 30,000 cells was placed onto the CAM. On day 13, tumor xenografts were clearly established and measurable, and the topical application of 10 µl imatinib solution was started. The experiment was terminated for ethical reasons between days 17 and 19 when the embryo grew too large and the surface of the CAM was not sufficiently smooth any more for microscopic observations.