

The Chick Embryo Chorioallantoic Membrane as a Model for *in vivo* Research on Anti-Angiogenesis

Domenico Ribatti*¹, Angelo Vacca², Luisa Roncali¹, Franco Dammacco²

¹Institute of Human Anatomy, Histology and Embryology and ²Department of Biomedical Sciences and Human Oncology, University of Bari Medical School, Bari, Italy



Abstract: Anti-angiogenesis, i.e. inhibition of blood vessel growth, is being investigated as a way to prevent the growth of tumors and other angiogenesis-dependent diseases. Pharmacological inhibition interferes with the angiogenic cascade or the immature neovasculature with synthetic or semi-synthetic substances, endogenous inhibitors or biological antagonists. The chick embryo chorioallantoic membrane (CAM) is an extraembryonic membrane commonly used *in vivo* to study both new vessel formation and its inhibition in response to tissues, cells, or soluble factors. Angiogenesis or anti-angiogenesis is evaluated quantitatively or semiquantitatively. The fields of application of CAM in the study of anti-angiogenesis, including our personal experience, are illustrated in this paper.

INTRODUCTION

Angiogenesis is a feature of embryonal development and in several physiological and pathological conditions, including rheumatoid arthritis, psoriasis, tumor growth and metastasis, diabetic retinopathy and age-related macular degeneration [1]. It appears to depend on the balance of several stimulating and inhibiting factors [2]. Angiogenesis-dependent diseases are controlled by using chemotherapy, immunotherapy and radiation therapy to inhibit the stimulating or stimulate the inhibiting factors.

Anti-angiogenesis, i.e. inhibition of blood vessel growth, as a way of treating primary tumors and reducing their metastases, was first proposed by Judah Folkman in 1971 [3]. Angiogenesis inhibitors are described as class 1 (specific and semi-specific) and class 2 (non-specific), depending on whether they inhibit proliferation and/or migration of endothelial cells only, or are also toxic for tumor cells [4]. About 20 inhibitors are currently being tested in human trials: most are in early phase I or II clinical studies; some are in or entering phase III testing [5].

The classical assays for studying angiogenesis *in vivo* include the hamster cheek pouch, the rabbit ear chamber, dorsal skin and air sac, the chick embryo chorioallantoic membrane (CAM) and the iris and avascular cornea of the rodent eye [6].

Several new models have recently been introduced including subcutaneous implantation of various three-dimensional substrates such as polyester sponges, polyvinyl-alcohol foam discs covered on both sides with a Millipore filter (the disc angiogenesis system), and Matrigel, a basement membrane-rich extracellular matrix. The three most widely used assays are CAM, the rabbit corneal micropocket and the subcutaneous implants. The main advantages of the CAM assay are its low cost, simplicity, reliability, lends itself to large-scale screening, which are important determinants of the choice of a method.

CHICK EMBRYO CHORIOALLANTOIC MEMBRANE

The CAM is an extraembryonic membrane formed on day 4 of incubation by fusion of the chorion and the allantois. Since it mediates gas exchanges with the extraembryonic environment until hatching, it has a very thick capillary network that forms a continuous surface in direct contact with the shell. Rapid capillary proliferation continues until day 11; the mitotic index then declines just as rapidly, and the vascular system attains its final arrangement on day 18, just before hatching [7].

IN OVO METHOD

Fertilized White Leghorn chicken eggs are placed in an incubator as soon as embryogenesis starts and are kept under constant humidity at 37°C. On day 3, a square window is opened in the shell after removal of 2-3 ml of albumen to detach

*Address correspondence to this author at the Institute of Human Anatomy, Histology and Embryology, University of Bari Medical School, Policlinico, Piazza G. Cesare, 11, I-70124 Bari, Italy; Fax +39.80.5478309; E-mail: ribatti@anatomia.uniba.it

the CAM from the shell. The window is sealed with a glass and incubation goes on until the day of experiment [8].

IN VITRO UTILIZATION

The embryo and its extraembryonic membranes are transferred to a Petri dish on day 3 or 4 of incubation. CAM develops at the top as a flat membrane and reaches the edge of the dish to provide a two-dimensional monolayer onto which multiple grafts can be placed because the entire membrane can be seen [9].

TESTING SUBSTANCES

The substance is soaked in inert synthetic polymers laid upon the CAM: Elvax 40 (ethylene-vinyl acetate copolymer) and hydron (a poly-2-hydroxyethyl-methacrylate polymer) are commonly used. Both polymers were first described and validated by Langer and Folkman [10]. They are biologically inert and polymerize in the presence of the test substance, allowing its sustained release at constant rates (nanograms to micrograms). When they are combined with an anti-angiogenic substance, the vessels become less dense around the implant and eventually disappear.

Alternatively, a fluid substance can be inoculated directly into the cavity of the allantoic vesicle, so that its activity will develop over the whole vascular area [11]. The anti-angiogenic response affects the CAM vessels as a whole.

In Nguyen *et al.* method [12], a collagen gel is conjugated with the test substance and placed between two parallel nylon meshes. The "sandwich" is then placed upon the CAM on day 8 of incubation. This method quantifies the new blood vessels growing vertically into the collagen gel as a percentage of the squares in the top mesh containing a vessel. Since histologic analysis is not required, a large number of compounds can be screened. The effect of an inhibitory substance (placed on the bottom mesh) is quantified by calculating the inhibition of the vasoproliferative response induced by an angiogenic factor, such as fibroblast growth factor-2 (FGF-2). One of the major advantages of the CAM assay is the use of various stimulators alone or in combination with an anti-angiogenic agent to examine the effectiveness of an inhibitor.

We have also described a new quantification method in which gelatin sponges are implanted on top of the growing CAM on day 8 [13]. Blood vessels growing vertically into the sponge and at

its boundary with the CAM mesenchyme are counted morphometrically on day 12. The gelatin sponge is also suitable for the delivery of cell suspensions onto the CAM. Furthermore, it is well tolerated and very little, if any, inflammatory reaction occurs. A common problem in the CAM assay is maintenance of the test substance at the site of administration. In the gelatin sponge/CAM assay, it is held within the graft and this adheres firmly to the CAM surface.

The CAM may also be used to verify the ability to inhibit the growth of capillaries by implanting tumors onto the CAM and by comparing tumor growth and vascularization with or without the administration of the anti-angiogenic substance [8].

SEMIQUANTITATIVE EVALUATION OF THE ANTI-ANGIOGENIC RESPONSE

Two independent observers determine the radius of the growth inhibition zone as 0-4 grades of vessel growth from the center of each disk to the furthest contiguous area in which tertiary vessels are absent. Zones with a radius greater than 1 mm are interpreted as evidence of significant inhibition of angiogenesis [14].

QUANTITATIVE EVALUATION OF THE VASOPROLIFERATIVE RESPONSE

Vessel density is quantified by morphometric evaluation of histologic CAM sections fixed at regular intervals after implantation. The total number of vessels in 6 randomly chosen fields are counted. Vessel density is evaluated planimetrically [15] with a 12-line x 12-line reticule inserted in the eyepiece of a photomicroscope. The total number of intersection points in 6 randomly chosen fields occupied by transversally sectioned microvessels 3 to 10 μm in diameter are counted. Vessel number and density are determined by two independent observers and processed statistically.

THE LIMITATIONS OF CAM

The major disadvantage of CAM is that it already contains a well-developed vascular network and the vasodilation that invariably follows its manipulation may be hard to distinguish from the effects of the test substance. Another limitation is nonspecific inflammatory reaction from the implant is that the histologic study of CAM sections demonstrates the presence of perivascular inflammatory infiltrate together

with any hyperplastic reaction of the chorionic epithelium. Nonspecific inflammatory reactions are much less frequent when the implant is made very early in CAM development and the host's immune system is relatively immature [16].

Another drawback is that polymers often do not adhere to the CAM surface. Folkman has suggested to hydrate test substance with 5 µl H₂O on a sterile coverslide glass, which is then turned over and placed on the CAM on day 9-10 [17]. Saline solutions cannot be employed because the hyperosmotic effect of crystal salts damages the chorion epithelium and induces fibroblast proliferation [18]. The substance must thus be used at concentrations of picograms to micrograms, as higher concentrations would cause this hyperosmotic effect [19].

Finally, it might emphasize that species-specific differences might arise if, for example, one attempt to test the effects of high affinity antibodies generated against human surface antigens. However, to circumvent this drawback, it is useful to perform the experiments early in the CAM development, since at that time the host's immune system is relatively immature [16].

TESTING ANTI-ANGIOGENIC SUBSTANCES IN THE CAM ASSAY (TABLE 2)

Angiogenesis is a complex multistep process and as such presents a number of key targets for therapeutic intervention. The broad mechanism, by which anti-angiogenic substances are through to work, are listed in (Table 1).

Table 1. Mechanism of Action of Anti-angiogenic Substances

Interference with angiogenic stimulators
Interference with angiogenic receptors
Interference with the extracellular matrix
Interference with the control of angiogenesis by hypoxic signaling
Interference with proteolysis
Vascular targeting

Table 2. Studies Demonstrating the Anti-angiogenic Activity of various Substances in the CAM Assay

Substance	Reference
VEGF-165 or VEGF-121 DT 385 (Diphtheria toxin)	21-22
Anti-FGF-2 antibody	24
Anti-angiogenin antibody	26-30
Anti-PIGF-1 antibody	32
Interleukin-2	33
Angiostatin	36
Endostatin	37
Steroids and heparin	39-42
Heparan sulfate	43
Protamine sulfate	44-45
Platelet factor-4	46
Heparanase inhibitors	48
Pentosan poly sulfate	49
GM 1474	50
Non- or low-sulfated saccharides	51
Inhibitor of arylsulfatase	55
Sulfated polysaccharide-peptidoglycan	56
Alpha, beta, gamma-cyclodextrin	57
Suramin	58-62
Spirolactone	63
Tyrosine kinase inhibitors	64-67
Antagonists of adhesion molecules	68-72
Matrix metalloproteinase inhibitors	73-74

(Table 2). contd....

Substance	Reference
Somatostatin	75-78
Nitric oxide	79-81
Anticancer agents	82-88
Hormones	89-90
Antibiotics	91-95
Cartilage	96-101
Thalidomide	102
Cyclosporin	103

To assess anti-angiogenic effects, noninvasive methods, including quantitation of angiogenic growth factors in serum and urine may be also used.

The more promising anti-angiogenic substances belong to the category of naturally occurring inhibitors include angiostatin and endostatin. They are highly specific for activated endothelial cells, have low toxicity and do not cause immunological response.

Antibodies to Angiogenic Stimulators

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF), is a heparin-binding angiogenic factor with endothelial target specificity [20]. The **VEGF-165 or VEGF-121 DT 385 (Diphtheria toxin)** conjugate blocks FGF-2-induced angiogenesis in the CAM [21-22].

Fibroblast growth factors (FGFs) are a family of heparin-binding polypeptides. **FGF-2** exerts angiogenic activity *in vivo* and induces cell proliferation, protease production and chemotaxis in endothelial cells *in vitro* [23]. A rabbit polyclonal **anti-FGF-2 antibody** inhibits angiogenesis in the CAM [24].

Angiogenin is a polypeptide isolated for the first time from the culture medium of a human adenocarcinoma cell line [25]. A **monoclonal antibody** to human **angiogenin**, synthetic peptides corresponding to the C-terminal region of angiogenin and a peptide complementary to its receptor-binding site inhibits angiogenin-induced

neovascularization in the CAM. Replacement of His-13 and His-114 in the ribonucleolytic and angiogenic activities of angiogenin and human placental ribonuclease inhibitor abolishes angiogenic activity in the CAM [26-30].

Placental-derived growth factor (PlGF) is a dimeric angiogenic heparin-binding glycoprotein showing a high degree of sequence similarity to the VEGF (31). An affinity-purified **anti-PlGF-1 antibody** inhibits angiogenesis in the CAM [32].

Naturally Occurring Inhibitors of Angiogenesis

Interleukin-2 (IL-2) has a slight effect on angiogenesis *in vivo* in the rabbit cornea model [33]. IL-2 inhibits angiogenesis in the CAM in a dose-dependent manner [34].

Angiostatin, a specific inhibitor of endothelial cell proliferation, is an internal fragment of mouse plasminogen, comprising the first four disulfide-linked kringle domain [35]. It inhibits angiogenesis in a number of primary and metastatic tumors [36].

Endostatin is a C-terminal fragment of collagen XVIII; it specifically inhibits endothelial cell proliferation and is a potent inhibitor of angiogenesis and tumor growth [37].

Angiostatin and endostatin have been demonstrated to induce tumor regression and tumor dormancy without drug resistance in several experimental models. Both inhibit angiogenesis in the CAM [36-37].

Synthetic and Small Molecular Weight Inhibitors

Sulfated analogs. A wide range of cellular functions including growth, morphology and migration are modulated by **heparin (HE)** and **heparan sulfate** [38]. HE consists of a mixture of polysulfated 6 to 20 kDa polysaccharides. Variations in the size of the polysaccharide chain and in the degree and distribution of sulfate groups contribute to a high degree of heterogeneity. HE alone may stimulate, inhibit or have no effect on angiogenesis *in vivo*. It binds angiogenic growth factors, including FGFs, VEGF, hepatocyte growth factor/scatter factor (HGF/SF) and the human immunodeficiency virus-1 transactivating factor tat. HE fractionated into low and high molecular weight species may inhibit or facilitate the binding of HE-binding growth factors to their receptors. Low molecular weight HE, for example, suppresses FGF-2-mediated angiogenesis more effectively. HE affects endothelial cell

proliferation and motility *in vitro* and modulates neovascularization *in vivo* when administered with certain **corticosteroids**.

CAMs treated with combinations of **angiostatic steroids** and **HE** reduce their vascularity and exhibit capillary **basement membrane fragmentation** and complete loss of fibronectin and laminin from the region of capillary involution. HE plus cortisone acetate and cortisone plus **hexasaccharide** inhibit angiogenesis, whereas HE, cortisone or hexasaccharide alone are non-anti-angiogenic [39-41]. **HE plus cortisone** induces a marked depression in the rate of collagenous protein biosynthesis in the CAM [42]. **HE** has an anti-angiogenic effect by itself, and an additive effect is obtained when it is combined with hydrocortisone. **Heparan sulfate** also has an anti-angiogenic effect, whereas **keratan sulfate, dermatan sulfate or chondroitin sulfate** have none [43].

Protamine and **platelet factor-4**, proteins that bind avidly to HE, inhibit angiogenesis. **Protamine sulfate** inhibits angiogenesis in the CAM [44-45]. Recombinant human **platelet factor-4** (rPF-4) inhibits angiogenesis in the CAM in a dose-dependent manner [46]. Both rPF-4 and an analog lacking affinity for HE (rPF4-241) inhibit angiogenesis in the CAM. The analog is inhibitory at lower concentrations than rPF4 and its inhibitory effects are not abrogated by the presence of HE [47].

Some of the most recent modifications of HE have focused on enhancing **heparanase** inhibitory activity. **Heparanase inhibitors** are anti-angiogenic in the CAM [48].

Pentosan polysulfate (PPS) is a **HE analog** used preclinically as an anticoagulant. It inhibits angiogenesis in the CAM [49].

GM 1474 is a low molecular weight **polysulfated oligosaccharide** that also binds to FGF-2. It inhibits angiogenesis in the CAM [50].

The anti-angiogenic effect of **non- or low-sulfated saccharides** is unaffected by the addition of **hydrocortisone**. **K5 polysaccharide**, its fragments down to octasaccharide size, and analogous N-acetylated fragments of heparan sulfate all show anti-angiogenic activity in the CAM. Hyaluronan, however, with the isomeric -(GlcA beta-1,3 GlcNA beta 1,4) (n) was inactive. The anti-angiogenic activity of -(GlcA beta-1,4 GlcNAc delta 1,4)-containing saccharides is potentiated by the presence of L-iduronic acid and one or two o-sulfate groups in the non-reducing terminal disaccharide unit [51].

The **heparan sulfate sulfheparoid** inhibits angiogenesis in the CAM [52]. A **sulfated polysaccharide-peptidoglycan complex (PS-4152)**, in the presence of **cortisone** or **tetrahydro S**, inhibits angiogenesis in the CAM [53-54].

A synthetic **inhibitor of arylsulfatase** (HNT), potentiates the anti-angiogenic activity of a mixture of **heparin** and **hydrocortisone** applied to the CAM in a dose-dependent manner. Hydrocortisone and HNT inhibit angiogenesis to the same extent as hydrocortisone and heparin. Preincubation of heparin with arylsulfatase causes 50% reduction in anti-angiogenic activity of heparin-steroid mixture applied to the CAM. This loss of activity is completely prevented by addition of HNT [55].

Angiogenesis induced by **Kaposi's sarcoma-derived spindle-shaped cells** in the CAM is blocked by a **sulfated polysaccharide-peptidoglycan** compound produced by bacteria [56].

Alpha, beta and **gamma-cyclodextrin** derivatives have been examined for their angiostatic activity in combination with **hydrocortisone** in the CAM [57].

The most thoroughly studied small-molecule sulfate inhibitor of angiogenesis is **suramin**, a polysulfonated naphthylurea used in the treatment of trypanosomiasis. Suramin alone shows anti-angiogenic activity in the CAM in a dose-dependent manner. It also potentiates the activity of the angiostatic steroids, hydrocortisone, cortisol-21-phosphate, 17-alpha-hydroxyprogesterone, tetrahydrocortisol, and tetrahydrocortexolone. HE decreases its angiostatic activity. **Eriochrome black T** (EBT), structurally related to suramin and suramin analogs, are more potent and less toxic than suramin in the CAM [58-62].

Steroids, flavinoids and steroid conjugates. Steroids are among the first small-molecules that show an anti-angiogenic effect *in vivo*. **Spirolactone** is an orally active, renal aldosterone antagonist used to treat hypertension, congestive heart failure and other diseases. It inhibits angiogenesis in the CAM [63].

Tyrosine kinase inhibitors. Protein tyrosine kinases are involved in induction of angiogenesis. **Staurosporine** and **erbastatin** inhibit angiogenesis in the CAM [64-65]. A series of compounds, originally studied as potential **PKC inhibitors**, including **diaminoanthraquinone NSC 639666**, inhibit angiogenesis in the CAM [66]. **PD98059**, a MEK inhibitor, inhibits FGF-2-induced angiogenesis in the CAM [67].

Adhesion molecules. The role of adhesion molecules (**selectins, immunoglobulin supergene family, cadherins and integrins**) in angiogenesis has been established. Analogs of the selectin ligand Sialyl Lewis X inhibited angiogenesis in the CAM [68]. **Integrin** $\alpha_v\beta_3$ allows endothelial cells to interact with a wide variety of extracellular matrix components. Endothelial cells exposed to growth factors or those undergoing angiogenesis express high levels of **Cyclic peptide or monoclonal antibody (LM 609)** against $\alpha_v\beta_3$ inhibits basal and TNF- α induced angiogenesis in the CAM. **Triflavin**, a member of the disintegrin family, inhibits TNF- α induced angiogenesis in the CAM. **Nonpeptide integrin antagonists** inhibit angiogenesis in the CAM [69-72].

Matrix metalloproteinase (MMP) inhibitors. MMPs are a series of zinc-requiring proteolytic enzymes, that are secreted in latent pro-enzyme form and are involved in remodeling and degradation of extracellular matrices. To the extent that proteolysis is an important component of angiogenesis, it can be argued that inhibitors of proteolytic activity should inhibit neovascularization. **Tissue inhibitor of MMP-3** inhibits FGF-2 induced angiogenesis in the CAM assay [73]. A **fragment of MMP-2 (PEX)**, a non-catalytic C-terminal hemopexin-like domain of MMP-2 with integrin binding activity, inhibits MMP-2 activity in the CAM, where it inhibits angiogenesis and tumor growth [74].

Miscellaneous Agents

Somatostatin and its analogs seem to be active in the inhibition of certain tumors. **Somatostatin analogs SM 201-995, RC-160 and octreotide acetate** inhibit angiogenesis in the CAM in a dose-dependent manner and show statistically significant inhibition of neovascularization when compared to native somatostatin 14. Furthermore, octreotide inhibits CAM neovascularization by human MCF-10A (int-2) mammary cells secreting FGF-3 [75-78].

Nitric oxide (NO) is an endogenous mediator released from a variety of cell types including endothelial cells, smooth muscle cells, platelets, macrophages and nerve cells of the peripheral and central nervous system. The nitrovasodilators **sodium nitroprusside (NaNP), isosorbide mononitrate (ISMN) and dinitrate (ISDN)**, which release NO spontaneously, and the amino-acid **L-arginine**, inhibit angiogenesis in the CAM. Furthermore, NaNP, ISMN and ISDN completely reverse the angiogenic effect of alpha-thrombin and the protein kinase C (PKC) activator 4-beta-phorbol-12-myristate-13 acetate [79-81].

Anticancer agents. Most anticancer agents are screened for their antiproliferative and differentiation-inducing activity on tumor cells, but not for their differential effects on vascular endothelium. Several cytostatic agents such as **doxorubicin (daunorubicin, epirubicin), mitoxantrone, etoposide, vincristine and vinblastine** are angiostatic in the CAM [82-83].

Immunoconjugate of doxorubicin on the galactose residues of a monoclonal antibody, specific for the tumor-associated carcino-embryonic antigen induces a reduction of tumor-induced angiogenesis and tumor progression in the CAM [84]. Antitumor agent **titanocene dichloride** [85-86], **taxol** [87] and the antineoplastic ether lipid **S-phosphonate** [88] inhibit angiogenesis in the CAM.

Hormones. The non-steroidal antiestrogens, especially tamoxifen, have been extensively used in breast cancer therapy, since they compete with endogenous estrogens for the estrogen receptor. Many recent studies have shown that antiestrogens affect the activity of many growth factors of importance in the control of cell proliferation. **Partial estrogen antagonists, clomiphene, tamoxifen, nafoxidine** and the pure estrogen antagonists, **ICI 164,384 and ICI 182,780**, inhibit angiogenesis in the CAM in a dose-dependent manner [89]. **2-methoxyestradiol**, an endogenous metabolite of estradiol-17 beta, inhibits FGF-2 induced angiogenesis in the CAM [90].

Antibiotics. Some antibiotics have anti-angiogenic properties. **TNP-470 (AGM-1470)**, a synthetic analog of fumagillin isolated from *Aspergillus fumigatus*, is a potent angiogenesis inhibitor *in vitro* and acts by preventing the entry of endothelial cells into the G1 phase. Locally administered TNP-470 [91] and medium-chain triglyceride (MTC) of TNP-470 [92] inhibit angiogenesis in the CAM. **FR-111142**, a new angiogenesis inhibitor [93] produced by the fungus *Scolecobasidium Arenarium* F-2015, **neomycin**, an **aminoglycoside antibiotic** [94], and **depudecin**, a **microbial metabolite** [95], inhibit angiogenesis in the CAM.

Cartilage. **Cartilage implants** inhibit basal angiogenesis in the CAM and angiogenesis induced by implants of Walker carcinoma or tumor angiogenesis factor (TAF). A factor in conditioned medium of rabbit costal **chondrocytes** inhibits angiogenesis induced in the CAM by B16 melanoma and by tumor transplants [96-97]. An angiogenesis inhibitor, isolated from the conditioned media of scapular chondrocytes, is angiostatic in the CAM [98]. Conditioned medium from a clonal **human chondrosarcoma cell line**, inhibits angiogenesis induced in the CAM by B16 melanoma [99]. A potent angiogenesis inhibitor,

U995, purified from the cartilage of the blue shark, inhibits TNF- induces angiogenesis in the CAM [100]. Purified recombinant human **chondromodulin-1** (ChM-1), purified from fetal cartilage, inhibits angiogenesis in the CAM [101].

Thalidomide, a well-known, potent teratogen inhibits angiogenesis in the CAM [102].

Cyclosporin is mainly known as immunosuppressive agent and is widely used in organ transplantation. It inhibits angiogenesis in the CAM [103].

CONCLUDING REMARKS

CAM is widely utilized as an *in vivo* system to study anti-angiogenesis. The rabbit cornea pocket assay [6] is used just as often as an *in vivo* system. CAM, however, offers the advantage of being relatively inexpensive and lends itself to large-scale screening, while the very few restrictions to its use are essentially due to nonspecific inflammatory reactions and to the presence of pre-existing vessels which make it difficult to determine the true extent of anti-angiogenesis.

ACKNOWLEDGEMENTS

This work was supported in part by grants from Associazione italiana per la Lotta al Neuroblastoma, Genoa; Associazione Italiana per la Ricerca sul Cancro, Milan, and Ministero dell'Università e della Ricerca Scientifica, Rome, Italy.

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