Chapter 12
The Chick Embryo Chorioallantoic Membrane Assay

Domenico Ribatti

12.1 Introduction

The chick embryo chorioallantoic membrane (CAM) is an extraembryonic membrane involved in gas exchange through a dense capillary network [1]. Due to its extensive vascularization, the CAM has been used to study the morpho-functional aspects of angiogenesis *in vivo* and to investigate the activity of pro-angiogenic and anti-angiogenic molecules [1]. Moreover, due to the lack of a fully developed immunocompetence system in the chick embryo, the CAM represents an ideal host tissue for tumor engrafting [2].

The allantoic vesicle enlarges very rapidly from days 4 to 10: the CAM surface increases from 6 cm^2 at day 6 to 65 cm^2 at day 14. In this process the mesodermal layer of the allantois fuses with the adjacent mesodermal layer of the chorion to form the CAM. An extremely rich vascular network connected to embryonic circulation by the allantoic arteries and veins develops between the two layers. Immature blood vessels scattered in the mesoderm grow very rapidly until day 8, giving rise to a capillary plexus, associated with the overlaying chorionic epithelium and mediating gas exchange with the outer environment. Capillary proliferation continues until day 10; then, the endothelial cell mitotic index declines and the vascular system attains its final arrangement on day 12 [3]. Besides sprouting angiogenesis that characterizes the early phases of CAM development, late CAM vascularization is supported by intussusceptive microvascular growth in which capillary network increases its complexity and vascular surface by insertion of transcapillary pillars [4].

D. Ribatti (✉)
Department of Basic Medical Sciences, Neurosciences and Sensory Organs, University of Bari Medical School, Piazza Giulio Cesare, 11, Policlinico, 70124 Bari, Italy

National Cancer Institute "Giovanni Paolo II", 70124 Bari, Italy
e-mail: domenico.ribatti@uniba.it

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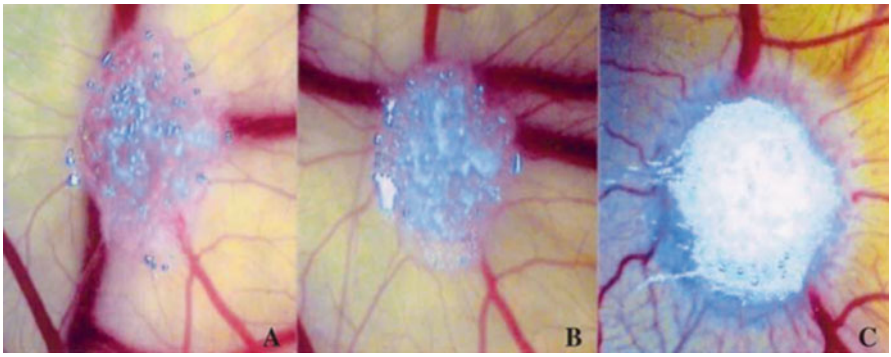

Fig. 12.1 Time-course of the macroscopic appearance of a chorioallantoic membrane implanted at day 8 (**a**) with a sponge loaded with 18,000 plasma cells of an active multiple myeloma patient. Note that, whereas on day 9 (**b**) no vascular reaction is detectable, in day 12 (**c**) numerous allantoids vessels develop radially towards the implant in a "spoked-wheel" pattern (Reproduced from Ribatti et al. [8])

Many protocols have been proposed to deliver macromolecules and low molecular weight compounds onto the CAM by using as vehicles silostatic rings, methylcellulose discs, silicon rings, filters, and plastic rings collagen and gelatin sponges. The gelatin sponge is also suitable for the delivery of cell suspensions onto the CAM surface and the evaluation of their angiogenic potential [5]. As compared with the application on the CAM of large amounts of a pure recombinant angiogenic cytokine in a single bolus, implants of cells overexpressing angiogenic cytokines enables a more physiological continuous delivery of growth factors.

Besides *in ovo* experimentation, a number of shell-less culture techniques have been devised, involving cultures of avian embryos with associated yolk and albumin outside of the eggshell. Shell-less cultures facilitate experimental access and continuous observation of the growing embryo.

An angiogenic response occurs 72–96 h after stimulation in the form of increased vessel density around the implant, with the vessels radially converging toward the center like spokes in a wheel (Fig. 12.1). Conversely, when an angiostatic compound is tested, the vessels become less dense around the implant and eventually disappear (Fig. 12.2).

Several semiquantitative and quantitative methods are used to evaluate the extent of vasoproliferative response or angiostatic activity at macroscopic and microscopic levels.

Many techniques can be applied within the constraints of paraffin and plastic embedding, including histochemistry and immunohistochemistry. Electron microscopy can also be used in combination with light microscopy. Moreover, unfixed CAM can be utilized for biochemical studies, such as the determination of DNA, protein and collagen content, and for reversal-transcriptase polymerase chain reaction (RT-PCR) analysis of gene expression. Finally, the study of intracellular signaling pathways mediating the angiogenic response to growth factors and cytokines have been successfully performed.

The main advantages of the CAM *in vivo* assay are the high embryonic survival rate, its low cost, simplicity, reproducibility and reliability. The major disadvantage

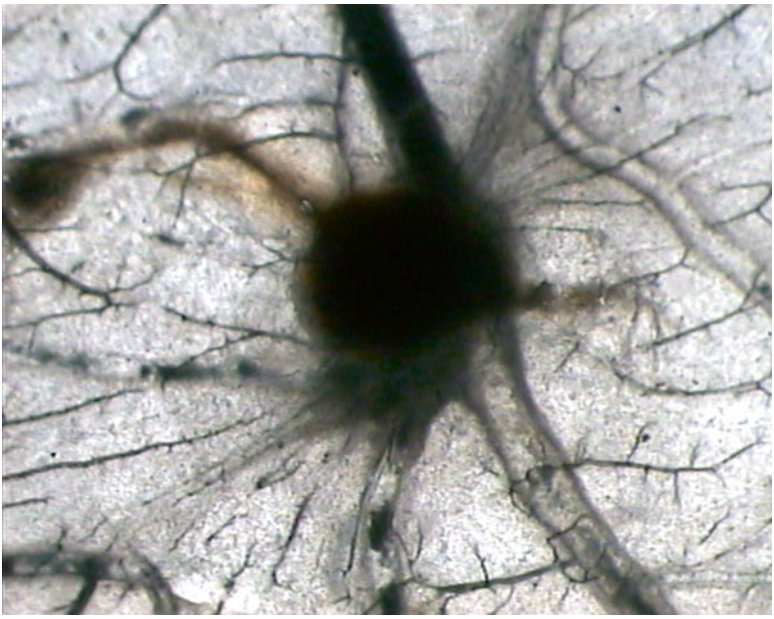

Fig. 12.2 Inhibitory effect of the urotensin receptor antagonist Palosuran on the angiogenic effect induced by urotensin II on the chorioallantoic membrane (Reproduced from Spinazzi et al. [9])

is that the CAM already contains a well-developed vascular network and the vasodilatation that follows its manipulation may be hard to distinguish from the effects of the test substance. Moreover, real neovascularization can hardly be distinguished from a falsely vascular density due to the rearrangement of existing vessels that follows contraction of the membrane [6]. Another limitation is nonspecific inflammatory reactions that may develop as a result of grafting, inducing a secondary vasoproliferative response [7]. Species-specific differences and the lack of avian-specific reagents (as well as limited genomic information) may represent other disadvantages. However, in the last years retroviral, lentiviral, and adenoviral vectors have been used to infect the CAM (as well as the whole chick embryo), leading to the expression of the viral transgene. This allows the long-lasting presence of the gene product that is expressed directly by CAM cells, and makes feasible the study of the effects of intracellular or membrane-bound proteins as well as of dominant-negative gene products.

12.2 Methodology

12.2.1 Reagents

1. White chicken egg obtained at day 1–2 postlaying (see Note 1).
2. 70 % (vol/vol) ethanol in dH_2O.
3. Sterile routine tissue culture medium (MEM Amino Acid Solution, Sigma).
4. Sterilized gelatin sponges (Gelfoam, Upjohn).

5. Angiogenic molecule (dissolved in 1–3 μl of sterile routine tissue culture medium at doses ranging between 10 and 500 ng per implant) or tumor cell suspension (from 1×10^4 to 6×10^6 cells per sponge resuspended in 3–4 μl of sterile routine tissue culture medium).
6. Bouin's fluid solution.
7. Standard solutions for paraffin embedding.
8. Toluene.
9. Embedding paraffin (Tissue-Tek VIP, electron Microscopy Science).
10. Toluidine blue 0 (Sigma; use a 0.5 % (vol/vol) aqueous solution).

12.2.2 Equipment

1. Egg incubator (Kemps Koops), 37 °C and 60 % humidity.
2. 25- or 26-G hypodermic needles and 1–ml syringes.
3. Curved-tip forceps.
4. Small dissecting scissors.
5. Transparent tape or glass coverslip, approximately 10×10 mm.
6. Microtome.
7. Stereomicroscope (Olympus, Italia).
8. Double-headed light microscope (BX51, Olympus, Italia), including a square mesh insert consisting of 12 lines per side for each eyepiece.

12.3 Methods

12.3.1 CAM Samples Preparation

1. Sterilize all instruments in 70 % ethanol before use.
2. Clean the fertilized white chicken eggs with 70 % ethanol and incubated at 37 °C and 60 % humidity in an egg incubator for 48 h (Note 2).
3. Aspirate 2–3 ml albumen from the egg using a 25- or 26-G hypodermic needle and 1-ml syringe at the acute pole of the egg on day 3 of incubation (Note 3).
4. After albumen removal, cut a square window into the shell with the aid of small dissecting scissors (Note 4).
5. On day 8 of incubation, open the window under sterile conditions with a laminar-flow hood and implant a 1 mm^3 sterilized gelatin sponge onto the CAM (Note 5).
6. Pipe the angiogenic molecule onto the sponge. Use a sponge containing vehicle alone as the negative control. Similarly, the gelatin sponge is suitable for the delivery of tumor cell suspensions onto the CAM surface and for the evaluation of their angiogenic potential. The sample may be a mixture of an angiogenic molecule and potential anti-angiogenic compounds (Note 6).

7. On day 12, fix the embryos and their membranes *in ovo* by pipetting 5 μl of Bouin's fluid solution onto the CAM surface and allowing the embryos to fix for 3 h at room temperature.
8. Cut the sponges and the underlying and immediately adjacent CAM portions with curved-tip forceps and transfer each specimen to a culture tube.
9. Dehydrate tissue samples in an ethanol series, clear them in toluene and immerse them in embedding paraffin for 2 h, according to manufacturer's instructions.
10. Using a microtome, cut 8-μm serial sections from each sample of paraffin-embedded CAMs in parallel to the surface of the membrane and stain the sections with a 0.5 % aqueous solution of toluidine blue for 1 min at room temperature.

12.3.2 Macroscopic Evaluation of the Vasoproliferative Response

On incubation day 12, macroscopic observation shows that the gelatin sponge treated with an angiogenesis stimulator is surrounded by allantoic vessels that develop radially towards the implant in a 'spoked-wheel' pattern (Note 7).

1. Analyzing the convergence of blood vessels toward the graft. For each egg, count the total number of macroscopic vessels that converge toward the graft under the stereomicroscope at 10 × magnification at different time points after implantation from day 8 to day 12. Express the data for each experimental group as the mean +/−1 standard deviation and obtain kinetics curves for proangiogenic or antiangiogenic stimuli compared with controls.
2. Analyzing variations in the distribution and density of CAM vessels next to the site of grafting. The intensity of the angiogenic response is scored under a stereomicroscope at regular intervals following the grafting procedure from day 8 to day 12 by means of a 0–5 scale of arbitrary values: 0 describes a condition of the vascular network that is unchanged with respect to the time of grafting; 1 marks a slight increment in vessel density associated to occasional changes in the course of vessels converging towards the grafting site; 2, 3, 4, and 5 correspond to a gradual increase in vessel density associated with increased irregularity in their course; a 5 rating also highlights strong hyperemia. A coefficient describing the degree of angiogenesis can also be derived from the ratio of the calculated value to the highest attainable value; thus, the coefficient's lowest value is equal to 0 and the highest value is 1.
3. Analyzing blood-vessel branching. A vascular index based on blood vessel branching may represent an alternative semiquantitative method to assess the vasoproliferative response. According to this procedure, all the vessels converging toward the implant and contained inside a 1 mm in diameter ring superposed to the CAM are enumerated: the ring is drawn around the implant in such a way that it will form an angle of less than 45° with a straight line drawn starting from the implant's center. Vessels branching outside the ring are scored as two, while those branching inside the ring are scored as 1.

12.3.3 Microscopic Evaluation of the Angiogenic Response

Quantitative evaluation of the angiogenic response, expressed as microvessel density, can be obtained by applying a morphometric method of 'point counting' on histological CAM sections. Briefly, two investigators simultaneously identify transversally cut microvessels (diameter ranging from 3 to 10 μm) among the gelatin sponge trabeculae with a double headed photomicroscope at × 250 magnification. A square mesh consisting of 12 lines per side, giving 144 intersection points, is inserted in the eyepiece. Ten sections are analyzed for each CAM specimen by observing every third section within 30 serial slides. For each section, six randomly chosen microscopic fields are evaluated for the number of intersection points occupied by microvessels. Then, mean values ± 1 standard deviation are determined for each CAM specimen. The microvessel density is expressed as percentage of intersection points occupied by microvessels. Statistically significant differences between the mean values of the intersection points in the experimental CAMs and control ones are determined by Student's *t* test for unpaired data.

At microscopic level, a highly vascularized tissue is recognizable among the trabeculae of the sponges treated with the angiogenesis stimulator. The tissue consists of newly formed blood vessels growing perpendicularly to the plane of the CAM, mainly capillaries with a diameter ranging from 3 to 10 μm within an abundant network of collagen fibers. In contrast, no blood vessels are present among trabeculae of the implants treated with vehicle alone. Otherwise, in the specimens treated with an angiogenesis inhibitor or with vehicle alone, few blood vessels are detectable around the sponge. Also, angiogenesis inhibitor causes the progressive regression of blood vessels distributed at the boundary between sponge and CAM mesenchyme, while they are still detectable in embryos treated with vehicle alone.

12.4 Notes

1. Chicken embryos from different vendors can vary significantly in their degree of vascularization and developmental status. Thus, consistent use of the same vender can decrease experimental variability.
2. Cleaning the egg shell before incubation will remove any debris associated with the outer surface and decrease the risk of infection. The eggs are very susceptible to mixed bacterial infections from feces-derived shell organisms, including *Aspergillus fumigatus*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. Furthermore, specific incubation conditions, including constant temperature and humidity, are of critical importance for proper vascularization and embryo survival.
3. This procedure creates a false air sac directly over the CAM, allowing its dissociation form the egg shell membrane. Make sure the needle opening is pointing away from the embryo during albumen aspiration. In addition, regular changes of needle and syringe limit the carryover of infection from egg to egg.

4. This window can be enlarged to approximately 10×10 mm. The underlying embryo and CAM vessels are revealed. Seal the window with transparent tape or a glass coverslip of the same dimension, and return the egg to the incubator. This step should be done in an enclosed area such as a laminar-flow hood to minimize the risk of infection. If large pieces of shell fall onto the CAM it may possible to remove these using fine forceps. It is probably best to discard eggs where pieces of shell have fallen onto the CAM and have not been removed very easily, as these eggs may develop false positive response due to inflammation.
5. For this purpose, the sponge is cut by hand with a blade and gently placed on top of the growing CAM. Use sterile gloves to minimize the risk of infection. Reject eggs with an excessively humid CAM; otherwise, sponges may float off during the incubation period. The CAM is an expanding membrane with vessels developing over its entire surface. It is preferable place the sponges between two large vessels and not place outer edges of the CAM.
6. Although most substances are soluble in water, small organic molecules, such as synthetic angiogenesis inhibitors, may require organic solvents. Ethanol, but not DMSO, can be used without adverse effects by soaking the gelatin sponge in the dissolved compound of interest and allowing the solvent to evaporate before implantation onto the CAM.
7. Two operators, preferably blinded to the sample identity should grade the angiogenic/antiangiogenic response. Different test substances can produce a range of different types of angiogenic response, such as a mixed response of microvascular growth and large vessel deformation/growth towards the point of application. Samples may also induce local bleeding and the presence and severity of these reactions should be noted, as the response may be secondary to the bleeding or inflammation.

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References

1. Ribatti D (2010) The chick embryo chorioallantoic membrane in the study of angiogenesis and metastasis. Springer, Dordrecht
2. Leene W, Duyzings MJM, Von Steeg C (1973) Lymphoid stem cell identification in the developing thymus and bursa of Fabricius of the chick. Z Zellforsch 136:521–533
3. Ausprunk DH, Knighton DR, Folkman J (1974) Differentiation of vascular endothelium in the chick chorioallantois: a structural and autoradiographic study. Dev Biol 38:237–248
4. Patan S, Haenni B, Burri PH (1993) Evidence for intussusceptive microvascular growth in the chicken chorio-allantoic membrane (CAM). Anat Embryol 187:121–130
5. Ribatti D, Nico B, Vacca A et al (2006) The gelatin sponge-chorioallantoic membrane assay. Nature Protoc 1:85–91
6. Knighton DR, Fiegel VD, Phillips GD (1991) The assay of angiogenesis. Prog Clin Biol Res 365:291–299

7. Jakob W, Jentzsch KD, Mauersberger B et al (1978) The chick embryo choriallantoic membrane as a bioassay for angiogenesis factors: reactions induced by carrier materials. Exp Pathol (Jena) 15:241–249
8. Ribatti D, De Falco G, Nico B et al (2003) In vivo time-course of the angiogenic response induced by multiple myeloma plasma cells in the chick embryo chorioallantoic membrane. J Anat 203:323–328
9. Spinazzi R, Albertin G, Nico B et al (2006) Urotensin-II and its receptor (UT-R) are expressed in rat brain endothelial cells, and urotensin-II via UT-R stimulates angiogenesis *in vivo* and *in vitro*. Int J Mol Med 18:1107–1112