Chapter 2

The Corneal Pocket Assay

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Abstract

The cornea in most species is physiologically avascular, and thus this assay allows the measurement of newly formed vessels. The continuous monitoring of neovascular growth in the same animal allows the evaluation of drugs acting as suppressors or stimulators of angiogenesis. Under anesthesia a micropocket is produced in the cornea thickness and the angiogenesis stimulus (tumor tissue, cell suspension, growth factor) is placed into the pocket in order to induce vascular outgrowth from the limbal capillaries. Neovascular development and progression can be modified by the presence of locally released or applied inhibitory factors or by systemic treatments. In this chapter the experimental details of the avascular cornea assay, the technical challenges, and advantages and disadvantages in different species are discussed. Protocols for local drug treatment and tissue sampling for histology and pharmacokinetic profile are reported.

Key words Angiogenesis, Capillary, Endothelial cell, Vascular endothelial growth factor, Fibroblast growth factor, Immunohistochemistry

1 Introduction

In order to develop and evaluate drugs acting as suppressors or stimulators of angiogenesis the continuous in vivo monitoring of angiogenesis is required. In this respect, there is the continuous work to provide animal models for quantitative analysis of in vivo angiogenesis [1]. The cornea assay consists in the placement of an angiogenesis inducer (tumor tissue, cell suspension, growth factor) into a micropocket made in the cornea thickness and the evaluation of vascular outgrowth from the peripherally located limbal vessels toward the stimulus. Since the cornea is initially avascular, this assay has the advantage of measuring only new blood vessels. Different antiangiogenic molecules have been found in the cornea as angiostatin, endostatin, interleukin-1 receptor antagonist, pigment epithelium-derived factor, and thrombospondin [2–4]. Recently, the preservation of the avascular phenotype of the cornea has been associated to high levels of soluble vascular endothelial growth factor receptor (sVEGR1), able to neutralize the VEGF-A present

Domenico Ribatti (ed.), *Vascular Morphogenesis: Methods and Protocols*, Methods in Molecular Biology, vol. 1214,
DOI 10.1007/978-1-4939-1462-3_2,

in the cornea [5]. Thus, vascularization occurring during different pathophysiological conditions is the result of the perturbed balance among redundant inhibitory mechanisms.

Gimbrone et al. firstly described the corneal assay in New Zealand white rabbits in 1974 [6]. Our group set up a series of modifications of the original method allowing the implant of multiple samples, including cell suspensions and tissue fragments. The assay was chosen for the absence of a preexisting vascular pattern and for the easy manipulation of the cornea and continuous monitoring of the neovascular growth. This technique, extensively used during the years, has been substantially modified to characterize angiogenesis inducers, to validate angiogenesis inhibitors, to study the interaction between different factors and the cellular, biochemical, and molecular mechanism of angiogenesis.

Refinement of drug formulation for local eye delivery and pharmacokinetic profile in eye components can be established.

In the following sections the experimental details and protocols of the avascular cornea assay are presented and its advantages and disadvantages in different species (rabbit, mouse, rat) are discussed.

2 The Rabbit Cornea Assay

First of all, the protocols and treatments must be approved by the local laboratory animal ethics board and the national agencies, according to the current laws (i.e., European Directive 2010/63/EU) since the surgical procedure requires general anesthesia.

The angiogenesis cornea assay is performed in albino rabbits (*see* **Note 1**) and requires the simultaneous presence of two qualified operators in all the steps (*see* **Note 2**).

2.1 Materials

2.1.1 Animals

New Zealand albino rabbits (Charles River, www.criver.com) of 1.5–2.5 kg (*see* **Note 3**).

2.1.2 Reagents and Drugs

1. Recombinant growth factors or drugs in water or phosphate buffered saline (PBS) or ethanol or methanol in highly concentrated solutions (0.1–1 mg/ml) (*see* **Note 4**).
2. Ethylene–vinyl acetate copolymer (Elvax-40) (DuPont de Nemours, Wilmington, DE, www.dupont.com) (*see* **Note 5**). Elvax-40 preparation and testing:
   (a) Weight 1 g of Elvax-40, wash it in absolute alcohol for 100-fold at 37 °C, and dissolve in 10 ml of methylene-chloride to prepare 10 % casting stock solution. Leave Elvax-40 in methylene chloride at 37 °C for 30–60 min to speed up solubilization.

   (b) Test the Elvax-40 preparation for its biocompatibility [7]. The casting solution is eligible for use if no implant performed with this preparation induces the slightest or histological reaction in the rabbit cornea.
3. Xylazine solution (20 mg/ml) (Xilor).
4. Zoletil-20, a combination of a dissociative anesthetic agent, tiletamine hypochloride, and a tranquilizer, zolazepam hypochloride (each at 10 mg/ml).
5. Benoxinate 0.4 %.
6. Tanax (T-61), an euthanasic mixture containing embutramide (200 mg/ml), mebenzonium iodide (50 mg/ml), and tetracaine hydrochloride (5 mg/ml).
7. Fixative: 4 % paraformaldehyde in PBS, pH 7.4.
8. Isopentane.
9. Liquid nitrogen.
10. OCT Tissue-Tek medium or similar.
11. Acetone.
12. Hematoxylin and eosin.
13. Phosphate buffered saline (PBS).
14. Hydrogen peroxide in PBS.
15. Bovine serum albumin (BSA).
16. Primary antibodies: for markers of neovascularization (anti-CD31 Ab, Dako), inflammation (anti-RAM11 Ab, Dako), and adhesion molecule (anti α5β1 integrin Ab, Chemicon).
17. Goat anti-mouse IgG (Sigma).
18. Mouse peroxidase anti-peroxidase (PAP, Sigma).
19. 3,3′ diaminobenzidine tetrahydrocloride (DAB, Sigma).
20. Aquatex medium (Merck).

2.1.3 Facilities and Equipment

1. Cell culture facility equipped with vertical laminar flow hood and autoclave.
2. Animal facility equipped with a sterile surgical room.
3. Disposable scalpel for ocular microsurgery (n° 10/11, Aesculap).
4. Teflon plate (10 × 10 cm), sterile forceps, silver spatula, microsurgery scissors, microspatula (*see* Fig. 1 for details).
5. 6 cm glass petri dishes.
6. Vacuum.
7. Latex dental dam for endodontic procedures (DentalTrey, www.dentaltrey.com).
8. Insulin syringes.

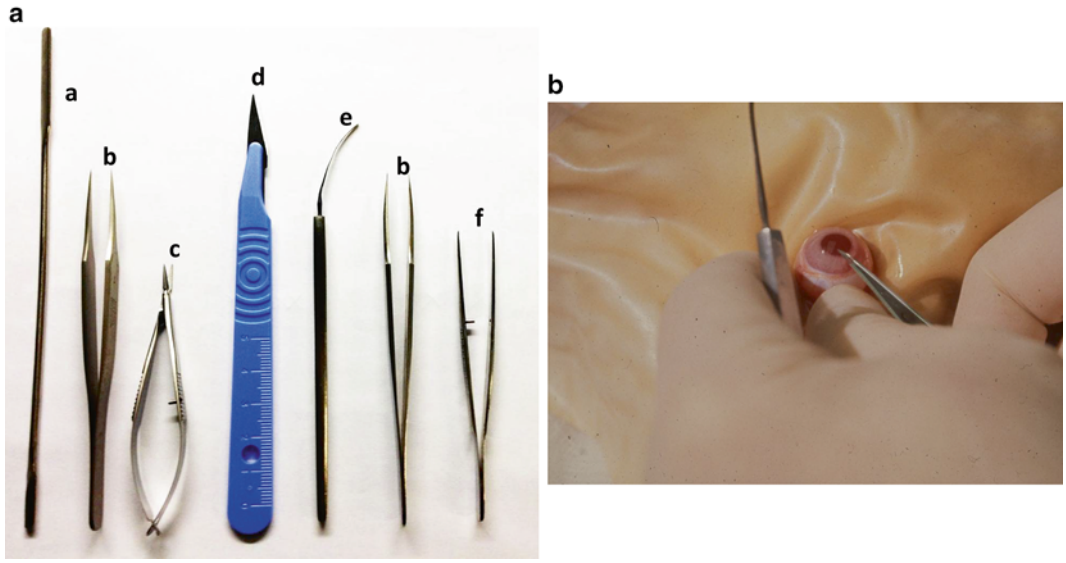

Fig. 1 (**a**) Instruments for pellet preparation and implant: (*a*) spatula, (*b*) Dumont tweezers, and (*c*) Vannas scissors for pellet preparation and manipulation; (*d*) disposable scalpel no. 10 and (*e*) pliable iris spatula for ocular microsurgery and micropocket creation; (*b*) Dumont Tweezer and (*f*) microforceps to keep open the edge of the corneal micropocket during pellet implantation. (**b**) Manipulation of animals for surgical implant. After induction of systemic anesthesia the eye is enucleated by the use of a clamp or a cotton swab and isolated through the use of a dental dam to allow the maintenance of a clean field during micropocket creation and pellet implant

9. Slit lamp stereomicroscope equipped with a digital camera.
10. Cryostat.
11. Slides and glassware for histology.
12. Microscope equipped with a digital camera.

2.2 Methods

2.2.1 Sample Preparation

The material under test can be in the form of slow-release pellets incorporating recombinant growth factors, cell suspensions, or tissue samples.

– Preparation of slow release pellets: In order to be implanted in the cornea, angiogenic factors (i.e., VEGF, FGF-2, cytokines or other molecules) have to be prepared in a semisolid state, enabling surgical implantation and gradual release of the factor in the polymer. Pellets (implants) bearing molecules to be tested are prepared under sterile conditions according to the following steps. A pre-determined volume of Elvax-40 casting solution is mixed by the use of stainless steel spatula with a given amount of the compound to be tested previously dried on a flat teflon surface. The polymer and the compound are homogeneously mixed under a laminar flow hood by the use of spatula. After drying, the film sequestering the compound is cut into $1 \times 1 \times 0.5$ mm pieces under a stereomicroscope by the

use of Vannas scissors and Dumont n. 5 tweezers. The pellets (in glass petri dishes) are left under vacuum at 4 °C overnight to remove residual solvent. Empty pellets of Elvax-40 are used as negative controls, while, depending on the experimental design, VEGF or FGF-2-containing pellets are used as positive controls (*see* **Note 6**).

- When testing the co-release of different molecules from the same pellet, the two substances are let to dry closely in the teflon plate and then incorporated in the same polymer preparation (*see* **Note 7**).
- Preparation of cell suspension: The intrinsic angiogenic potential due to different stages of tumor progression or to the expression of genes or gene products have been documented by our group as well as by others [8–11]. Prepare a cell suspension by trypsinization of confluent cell monolayers to a final dilution of $2–5 \times 10^5$ cells in 5 μl. When implanting cells, angiogenic response can be graded based also on the number of cell implanted into the corneal stroma.
- Preparation of tissue samples: Sample tissues from humans and experimental animals have been successfully implanted into the rabbit cornea to produce angiogenesis [12–15]. When tissues are tested, fragments are removed within 2 h from patients or animals and kept at 4 °C in complete medium. Samples of 2–3 mg are obtained by cutting the fresh tissue fragments under sterile conditions by the use of microdissection instruments under a stereomicroscope.

2.2.2 Surgery

1. Anesthetise animals with Xilor (0.5 ml, i.m.) followed by Zoletil (5 mg/kg i.m.) or alternatively sodium pentothal (10 mg/kg, i.v.) The deepness of anesthesia is checked as reflex to pressure (*see* **Note 8**).
2. Each eye is enucleated by the use of a dental dam (Fig. 1) and a local anesthetic (0.4 % benoxinate) is instilled just before surgery.
3. The pellet implantation procedure starts with a linear intrastromal incision, parallel to the corneoscleral limbus (linear keratotomy), using a surgical blade (disposable scalpel n. 10). The corneal pocket for the pellet implant is produced with a 1.5 mm pliable silver spatula with smooth edge blade in the lower half of the cornea (*see* **Note 9**).
4. Pellet implant: The implant is introduced through the keratotomy line, parallel to the corneal epithelium and under it, in the external third of the stroma, up to 2 mm from the limbus. One single pellet is selected from the petri dish using Dumont n. 5 tweezers and then introduced in the corneal pocket (Fig. 1). Microforceps are used to keep open the edge of the

cut. Locate the implant at 2 mm from the limbus to avoid false positives due to mechanical stress and to favor the gradient diffusion of test substances in the tissue, toward the endothelial cells at the limbal plexus.

5. When two factors are tested simultaneously, make two independent and parallel micropockets.
6. Cell or tissue implant: The pocket is produced with an enlarged base (4 mm) to allocate cell samples. A small amount (20–50 μl) of the aqueous humor can be drained from the anterior chamber with an insulin syringe to reduce corneal tension before cell or tissue implant.
7. By using a micropipette introduce 5 μl containing $2–5 \times 10^5$ cells in medium supplemented with 10 % serum in the corneal micropocket. When the overexpression of growth factors/inhibitors by stable transfection of specific cDNA is studied, one eye is implanted with transfected cells and the other with the wild type or vector transduced cell line. Suitable cell lines for these experiments are mammary carcinoma cells (MCF-7), lymphoma Burkitt's cells (DG75), Chinese hamster ovary cells (CHO) [9, 11, 16]. It might be necessary to evaluate the angiogenic potential of drug-treated cells. In these experiments cell monolayers are pharmacologically treated before the implant (18–24 h). One eye is implanted with treated cells and the controlateral with control cells [16, 17].
8. Tissue fragments are inserted in the corneal pocket with the aid of Dumont n. 5 tweezer. The angiogenic activity of tumor samples is compared with healthy tissue [14].
9. When drug solutions incompatible with Elvax polymerization and genes transduced by viral vectors have to be locally tested, microinjection of concentrated solutions is performed by the use of insulin syringes equipped with 30G needles. After the removal of aqueous humor, a volume of 10 μl is injected within the corneal stroma in the space between the limbus and the pellet implant [18].

2.2.3 Quantification of Neovascular Growth

1. Subsequent daily observation of the implants is made with a slit lamp stereomicroscope without anesthesia. The clinical evolution of the implants and of the ocular lesions are recorded and the presence of corneal reactions, such as redness, corneal edema, the intensity of the corneal cellular infiltrate, the total area of neovascularization are scored.
2. An angiogenic response is scored positive when budding of vessels from the limbal plexus occurs after 3–4 days and capillaries progress to reach the implanted pellet in 7–10 days. Implants that fail to produce a neovascular growth within 10 days are considered negative, while implants showing an inflammatory reaction are discarded.

3. During each observation the number of positive implants over the total implants performed is scored.
4. The potency of angiogenic activity is evaluated on the basis of the number and growth rate of newly formed capillaries, and an angiogenic score is calculated by the formula [vessel density × distance from limbus] [16, 19]. A density value of 1 corresponds to 0–25 vessels per cornea, 2 from 25 to 50, 3 from 50 to 75, 4 from 75 to 100, and 5 for more than 100 vessels. The distance from the limbus is graded (in mm) with the aid of an ocular grid.
5. To understand the mechanism of progression and/or regression by drug treatment, the two parameters (density and length) are considered separately, thus documenting the activity of treatment on endothelial cell proliferation (density) respect to elongation and organization (length).
6. The anterior ocular pole images are computer analyzed at fixed times on animals under anesthesia. An advanced video camera connected to a color video monitor and a computer with video-bluster and special capture software are used to record corneal responses. In order to extract the vascular tree from every image, the following graphic processing is required:
   - Adjustment of contrast and brightness, in order to highlight the vascular tree;
   - Image conversion in a gray scale format;
   - Image extraction of the vascular tree (skeletonization).

   Commercially available software (i.e., Corel Photo Paint and Corel Draw; Adobe Photoshop and National Institute of Health Image J1.38X) can be used for these purposes [20].

2.2.4 Histological Examination and Immunohistochemical Analysis

Depending on the experimental design, histological or immunohistochemical analysis of corneal sections can be performed at fixed times during angiogenesis progression or at the end of the observations [16].

1. Animals are sacrificed with intravenous injection of 0.5 ml of Tanax or sodium pentothal (bolus 30 mg/kg).
2. The corneas are removed, oriented, and marked (*see* **Note 10**), immediately frozen in isopentane cooled in liquid nitrogen for 10 s, and stored at −80 °C in OCT Tissue-Tek medium. If required, the cornea can be fixed it in paraformaldehyde
3. Seven-μm-thick cryostat sections are stained with haematoxylin and eosin and adjacent sections are used for immunohistochemical staining. After fixation in absolute acetone at −20 °C for 5 min, the sections are washed in phosphate buffered saline (PBS) and then treated with 1.5 % hydrogen peroxide in PBS for 8 min in order to perform quenching of endogenous

peroxidases. Aspecific binding sites are then blocked in 3 % bovine serum albumin (BSA) in PBS for 45 min. The sections are incubated overnight with the primary antibodies diluted in 0.5 % BSA in PBS.

4. Primary antibodies can be anti-CD31 Ab (Dako, 200 μg/ml) (marker of neovascularization), anti-RAM11 Ab (Dako, 1.2 μg/ml) (marker of inflammation) (anti α5β1 integrin Ab, Chemicon, 1:50) (adhesion molecule expressed in epithelial and endothelial cells). For co-localization studies, serial and adjacent sections can be labelled with different antibodies.
5. The sections are extensively washed in 0.5 % BSA in PBS and then incubated in goat anti-mouse IgG (Sigma,1:40) for 1 h. After washing in 0.5 % BSA in PBS, the sections are incubated in mouse peroxidase anti-peroxidase (PAP, Sigma 1:35) for 45 min. Immunoreaction is developed in 3,3′ diaminobenzidine tetrahydrocloride (DAB, Sigma) for 8 min. The sections are then extensively rinsed in dH_2O, counterstained in haematoxylin and mounted in Aquatex medium (Merck).
6. Sections are observed at the microscope (at 10–40× magnification) and digital images are taken.

2.3 Drug Treatment and Pharmacokinetics Studies

When performing drug treatments for ocular pathologies and to validate stimuli or signalling pathway, different approaches can be followed.

1. Eye drops: isotonic buffers (i.e., PBS without calcium and magnesium) at physiological pH can be used to dissolve drugs to be studied for their ability to modulate corneal angiogenesis. Depending on drug nature and half-life, eye drop treatments can be performed twice to five times a day, soon after an angiogenic stimulus has been implanted in the cornea stroma. Awake animals are immobilized in appropriate contention boxes. By the use of a sterile pipet, 100 μl of the drug solution is put in the subconjunctival space by pulling the lower lid. The eye is then kept close for at least 30 s to avoid liquid dispersion and drop out.
2. Ointment and gels: Simple eye ointment contains liquid paraffin (mineral oil) and wool fat (lanolin) in a yellow soft paraffin base (*see* **Note 11**). These ingredients produce a transparent, lubricating, and moistening film on the surface of the eyeball. Drug mixing is performed under hood and insulin syringes are prepared. 100 μl of ointment are poured in the subconjunctival space once or twice a day. Eyelids are closed and gently frictioned to form a film of the ointment or gel on the eye surface.
3. Intravitreal injections (30–50 μl/eye) can be also performed under general anesthesia to study drug stability in the vitreous and diffusion to retina or to the anterior chamber, to obtain data closely relate to human ocular pharmacology.

4. At fixed times after treatment started, following animal sacrifice, all the eye tissues (cornea, aqueous humor, lens, vitreous humor, retina) can be isolated and frozen in liquid nitrogen, and tissue homogenates assessed for drug distribution and metabolism.

2.4 Notes

1. Cornea has been found avascular in all strains examined so far. In albino rabbits the newly formed vessels are clearly visible on the background of the iris.
2. Operator skill for pellet manipulation, surgery, and monitoring of angiogenesis is required.
3. Body weight: in the range 1.8–2.5 kg for an easy handling and prompt recovery from anesthesia. Sex: except when hormone dependency of cells or tumors is a prerequisite of the experimental setting, males are used. Check with your animal facility and veterinary doctor whether only specific pathogen free (SPF) animals are admitted.
4. Sterility of materials and procedures is crucial to avoid non specific responses. DMSO should be avoided since incompatible with Elvax-40 polymerization and handling.
5. Polyvinylalcohol and Hydron can be used instead of Elvax-40. In our experience, a polymer of hydroxyethyl-methacrylate, gave less satisfactory results than Elvax-40.
6. Variability among growth factors in inducing angiogenesis has been found considering different angiogenic factors, different providers and batch of preparation. Usually the dose of VEGF (Fig. 2) or FGF-2 able to give positive angiogenic response varies in the range 200–400 ng/pellet.
7. When two factors are co-released from the same pellet, the advice is to check if drug release in vitro is modified respect to the single molecule.

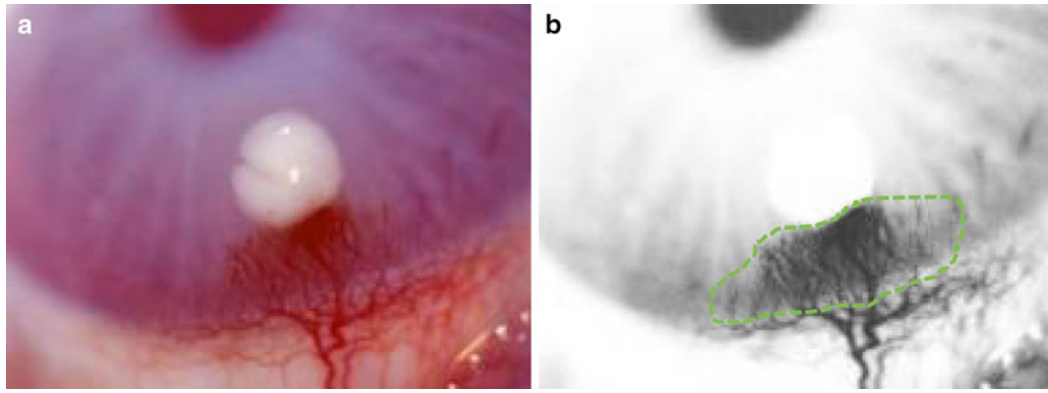

Fig. 2 Picture of neovascular growth induced by VEGF (200 ng/pellet) taken at day 10 (**a**). The newly formed vessels start form the limbal vasculature and progress toward the implanted stimulus. Panel **b** represents the same image processed for computerized image analysis

8. Immobilization (in appropriate contention box) during anesthetic procedure and observation is important to avoid self-induced injury.
9. Make the cut in the cornea in correspondence of the pupil and orient the micropocket toward the lower eyelid for an easy daily observation.
10. Before embedding in OCT Tissue-Tek medium, pellets should be removed and corneas sampled and marked (i.e., with a cotton thread) for subsequent orientation at the cryostat once embedded in OCT medium.
11. When using eye ointment, take into account that paraffin based preparations have to be used for short time (1 week) to avoid toxicity by excipients.

3 The Mouse Corneal Micropocket

The mouse cornea micropocket assay was firstly described by Muthukkaruppan and Auerbach [21].

1. Anesthetize animals with methoxyflurane.
2. Make a corneal micropockets in both eyes reaching within 1 mm of the limbus and pellets containing substances to be tested coated with Hydron (Interferon Science, New Brunswick, NJ) are implanted.
3. Use Hydron as a casting solution (12 % w/v) solution, prepared dissolving the polymer in absolute alcohol at 37 °C [7]. When peptides are tested, sucralfate (sucrose aluminum sulfate, Bukh Meditec, Copenhagen, Denmark) is added to stabilize the molecule and to slow its release from Hydron [22, 23].
4. The vascular response measured as the maximal vessel length and number of clock hours of neovascularization is scored at fixed time (usually on postoperative 5 and 7 days) using a slit-lamp biomicroscopy and photographed. To quantify the section of the cornea in which new vessels are sprouting from the preexisting limbal vessels, the circumference of the cornea is divided into the equivalent of 12 clock hours. The number of clock hours of neovascularization for each eye is measured during each observation.

4 The Rat Corneal Assay

1. Purified growth factors are combined 1:1 with Hydron as described by Polverini and Leibovich [24].
2. Pellets are implanted 1–1.5 mm from the limbus of the cornea of anesthetized rats (sodium pentobarbital, 30 mg/kg, i.p.).

3. Neovascularization is assessed at fixed days (usually 3, 5 and 7 days): animals are perfused with colloidal carbon solution to label vessels, eyes are enucleated and fixed in 10 % neutral buffered formalin overnight. The following day, corneas are excised, flattened, and photographed. A positive neovascularization response is recorded only if sustained directional growth of capillary sprouts and hairpin loops toward the implant is observed.

5 Advantages and Disadvantages in Different Species

5.1 Species

The rabbit size (1.8–2.5 kg) lets an easy manipulation of the animal; the eye may be easily extruded from its location for surgery manipulation and daily observation.

Rabbit cornea has been found avascular in all strains examined so far. In some strains of rats the presence of preexisting vessels within the cornea and the development of keratitis are serious disadvantages. Furthermore, rabbits are more docile and amenable to handling and experimentation than mice and rats. In case of inflammatory reactions, these are easily detectable in rabbits by stereomicroscopic examination as corneal opacity.

5.2 Measurements

In mice and rats it is possible to obtain time-point results. The evolution of the angiogenic response in the same animal is not recommended because each time the cornea is observed the animal has to be anesthetized. Experiments are made with a large number of animals and vessel growth during time can be visualized by perfusion with colloidal carbon solution in individual animals. Multiple observations are easily performed in rabbits, thus reducing the number of animals required for statistical evaluation. The use of slit lamp stereomicroscope and of awake animals allows the observation of newly formed vessels during time with prolonged monitoring, up to 1–2 months.

5.3 Different Experimental Procedures

In the rabbit eye, due to its wide area, stimuli in different forms can be introduced. In particular the activity of specific growth factors can be studied in the form of slow-release pellets [9, 25–27] and of tumor or non-tumor cell lines stably transfected for the overexpression of angiogenic factors [9, 16, 17]. Cells with double transfection can also be studied [9, 10]. The modulation of the angiogenic responses by different stimuli can be assessed in the rabbit cornea assay (a) by implanting single pellets releasing both the angiogenic stimulus and the inhibitor [28–30], (b) by implanting in the same cornea two pellets placed in parallel micropockets and releasing different molecules [31, 32], and (c) through the addition or removal of single pellets in multiple implants [31]. The implant of tumor samples from different locations can be

performed both in corneal micropockets and in the anterior chamber of the eye to monitor angiogenesis produced by hormone-dependent tissues or tumors (i.e., human breast or ovary carcinoma in female rabbits) and it allows the detection of both the iris and the corneal neovascular growth [15, 33].

5.4 Viral Vector Transduction

We have also explored the use of adenoviral vectors transducing inhibitors of the MAPK pathway. An example of the effect obtained by directly injecting the corneal tissue at the tip of the neovascular front [18] is shown in Fig. 3.

5.5 Drug Treatment

The effect of local drug treatment on corneal neovascularization may be studied in the form of ocular drops or ointment [34] or microinjection in the corneal thickness [18]. The effect of systemic drug treatment on corneal angiogenesis may be also evaluated [14, 16, 19]. However, when considering the size of the animals, systemic drug treatment in rabbits requires a higher amount of drugs than smaller animals.

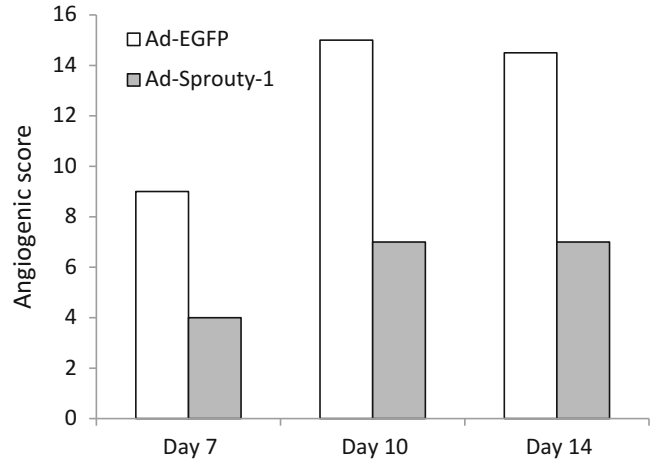

Fig. 3 Overexpression of endogenous MAPK inhibitor of angiogenesis by FGF-2. By way of an adenoviral vector (obtained in collaboration with G. Christofori), an endogenous MAPK inhibitory protein was transfected into the corneal tissue. Active endothelial cell proliferation was selected as a target of the treatment. Therefore when new vessels started to progress from the limbal plexus in the corneal stroma (at day 3 after implant of 200 ng/pellet of FGF-2), adenovirus (6×10^8 particles/eye) was microinjected under stereomicroscopic monitoring in the corneal tissue just above the tip of sprouting capillaries. Twenty microliters of Ad solutions was injected by the use of 30G gouge needle. Contralateral eyes were injected with the same number of Ad-EGFP. Injection was performed once and angiogenesis was followed during time for 2 weeks. Data are expressed as angiogenic score (mean of four implants) during time. The inhibition of FGF-2 activity and the neovascular progression were apparent immediately after treatment and persisted over time

Interestingly, the use of nude mice allows the study of angiogenesis modulation in response to effectors produced and released by tumors or tumor cell lines of human origin growing subcutaneously. Treatment of mice with antiangiogenic or antitumor drugs allows the simultaneous measurement of tumor growth and metastasis and corneal angiogenesis.

Acknowledgements

The work was supported by the Italian Ministry of University (MIUR), the Italian Association for Cancer Research (AIRC) to M.Z. and Istituto Toscano Tumori (ITT) to L.M.

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Vascular Morphogenesis
Methods and Protocols
Ribatti, D. (Ed.)
2015, XI, 272 p. 61 illus., 40 illus. in color., Hardcover
ISBN: 978-1-4939-1461-6
A product of Humana Press