THE CHICK CHORIOALLANTOIC MEMBRANE AS A MODEL TISSUE FOR SURGICAL RETINAL RESEARCH AND SIMULATION

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Purpose: We describe the use of chick chorioallantoic membrane (CAM) as a model system for the study of the precision and safety of vitreoretinal microsurgical instruments and techniques.

Methods: The CAM was prepared for experimentation with and without its inner shell membrane (ISM) attached for *in vivo* and *in vitro* experiments that simulated medical and surgical interventions on the retina.

Results: The CAM's ease of use, low cost, and anatomic structure make it a convenient model for surgical retinal and retinal vascular modeling.

Conclusion: While CAM has been used extensively in the past for ocular angiogenesis studies, we describe the tissue as a useful tool for a variety of other applications, including (1) testing of novel surgical tools and techniques for cutting and coagulating retina and its vasculature, (2) testing vessel cannulation and injection techniques, (3) angiographic studies, and (4) endoscopic surgery.

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Cancer biologists, developmental biologists, and ophthalmologists have described the chick chorioallantoic membrane (CAM) as a model system for studying development,[1] cancer behavior,[2–3] properties of biomaterials,[4] angiogenesis,[5–10] and photodynamic therapy.[11] We propose several new applications for CAM in the study of retina and its vasculature with respect to microsurgical interventions. Herein we describe the anatomic features of the CAM and some of the types of microsurgical interventions that can be tested on it.

The chick CAM, a part of the extraembryonic tissue, begins to develop 7 days after initial incubation from the fusion of the chorion and the allantois.[12] Structurally, the outer epithelial layer of the chorion is derived from the trophoblast, which opposes the allantois. This structure forms a supportive matrix for the extensive vascular network that courses through the CAM, analogous to the retina and its vasculature (Figure 1, inset). Overall, mature chick CAM (20–100 μm) and human retina (approximately 100–300 μm) are of roughly comparable thickness.[13] Mature chick CAM (incubation day 12 and on) can be divided into three anatomically distinct layers: (1) primary stratum; (2) capillary plexus, or blood sinus; and (3) a thin

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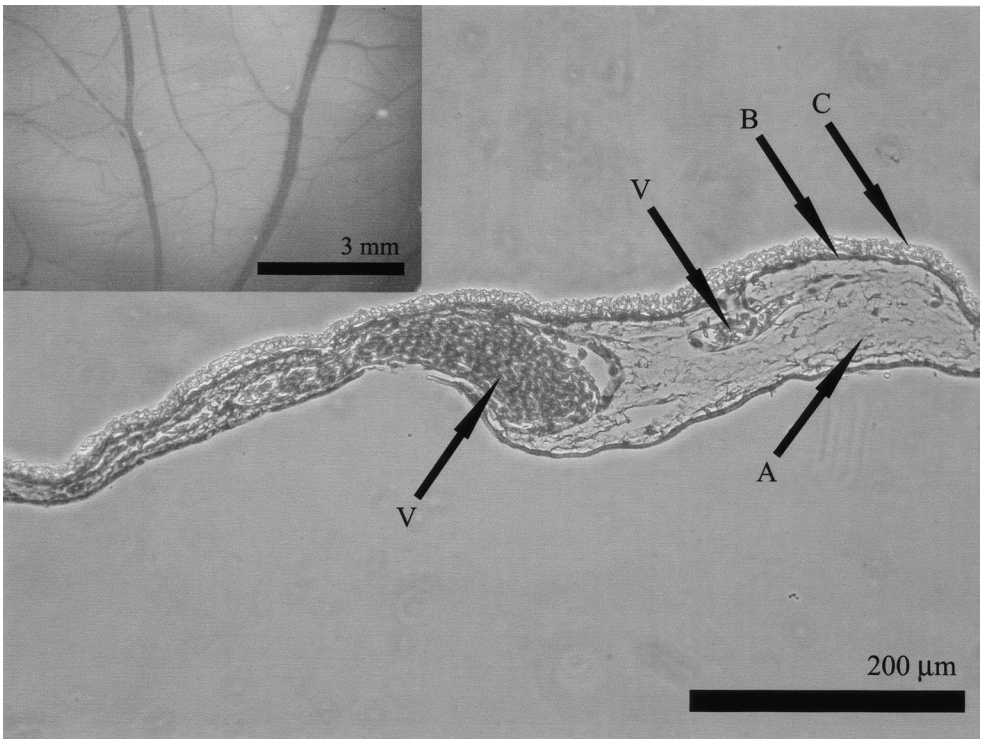

Fig. 1. Chick chorioallantoic membrane (CAM). Photograph of mature chick CAM (inset) demonstrating an arborizing network of vessels similar to retina. Histologic cross-section of mature chick CAM demonstrating large and small blood vessels (V), primary stratum (A), thin stratum (B), and inner shell membrane (ISM) (C). A capillary plexus/blood sinus exists between the primary (A) and thin (B) layers. Some erythrocytes in this plexus/sinus are faintly visible as circular bulges around the thin stratum (B).

stratum composed primarily of specialized chorionic epithelial cells that have presumably migrated above the capillary plexus/blood sinus and are involved in gas exchange and calcium absorption.[14] Immediately above and attached to the CAM thin layer is the inner shell membrane (ISM) (Figure 1).[15,16]

Closest to the developing embryo is the primary stratum. This is the thickest part of the CAM (approximately 20–100 μm thick), and the largest CAM vessels (up to approximately 1 mm in diameter) run immediately underneath and are anchored to this layer via thin, perivascular sheathes. Smaller vessels ($<$200 μm diameter) are embedded within the main layer.[17]

Immediately external to the primary layer of CAM is a capillary plexus, or blood sinus.[14] Above this sinus lies a thin stratum (approximately 1 μm) that is composed of four sublayers originally derived from the chorion (basement membrane, epithelial cell layer, peptidoglycan extracellular matrix, and basal lamina). The basement membrane lies in direct contact with the capillary plexus/blood sinus. During development, the epithelial cell layer's cytoplasm is progressively lost, giving these cells a thinned, vacuoled appearance. Directly above the basal lamina of the thin layer lies the ISM, an approximately 20 μm thick, acellular, calcium-rich membrane containing peptidoglycans and collagen types I, V, and X.[15,18] The thin layer's basal lamina contains extensive microvilli that penetrate into the ISM[12] and contain calcium transporters that transfer calcium stored in the ISM to the developing embryo via the extraembryonic tissue.[19–21]

Methods

Care and Preparation for Experimentation

Fertilized chick eggs are easily ordered, often do not require an extensive animal protocol (as long as they are used and disposed before 19 days), and are considerably less expensive than the live animals currently used in ophthalmic experimentation (e.g., rats, rabbits, cats, minipigs, dogs). Maintenance of eggs, which requires adequate humidity, a 37°C environment, and rotation every few hours, can all be performed in an inexpensive incubator.

To prepare CAM for a typical experiment, the eggshell is cracked and peeled away from the region over the air space that exists between the shell and the inner shell membrane (ISM) at one pole of the egg. This airspace can be visualized before the egg is cracked by holding the egg under intense light (e.g., from a surgical microscope). Once the opaque CAM-ISM dual layer is exposed, irrigation of the CAM-ISM with saline will cause the dual layer to become translucent, allowing for visualization of the CAM vasculature. Depending on the requirements of the proposed experiment, the ISM can be left in place or peeled away from the CAM with the use of fine forceps (Figure 2).

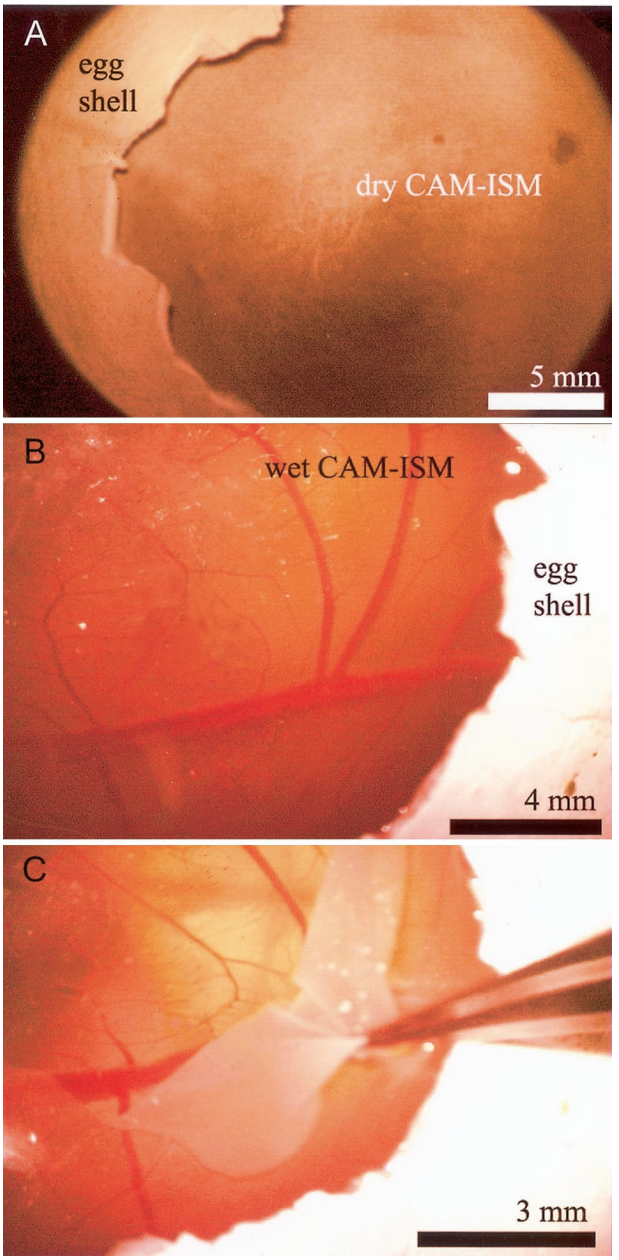

Fig. 2. Preparation of CAM. **A**, To prepare CAM for a typical experiment, the eggshell is cracked and peeled away from the region over the air space that exists between the shell and the ISM near one pole of the egg. **B**, Irrigation of the CAM-ISM with saline causes the dual layer to become translucent, allowing for visualization of the CAM vasculature. Experiments can be performed at this stage. **C**, The ISM can also be peeled away from the CAM with the use of fine forceps.

Since there is an extensive blood sinus/capillary bed between the CAM primary stratum layer and the ISM, peeling the ISM may cause temporary minor bleeding in some eggs. This bleeding, however, usually stops within 1 to 3 minutes. Introducing fresh saline into the air space will wash away the blood, providing a clean membrane for experimentation.

The type of CAM experiments described herein can optimally be performed between incubation days 12 and 18. Before day 12, the CAM vasculature has not adequately matured. After day 18, the embryo is large enough that its size and movements underneath the CAM can disrupt experimental maneuvers on the CAM. Additionally, standard animal protocols for chick embryos past day 18 require more complicated euthanasia techniques (chicks typically hatch between days 20 and 22).

Uses

Researchers interested in ocular angiogenesis, including antiangiogenic drugs and photodynamic therapy, have used CAM as an effective model system in the past.[6–11] We describe, to the best of our knowledge, four new uses for CAM in retinal research: (1) testing of novel surgical tools and techniques for cutting and coagulating retina and its vasculature; (2) testing vessel cannulation and injection techniques (especially for clot release); (3) angiographic studies; and (4) endoscopic surgery.

Testing of Novel Surgical Tools and Techniques for Cutting and Coagulating the Retina and Its Vasculature

The easy accessibility of CAM compared to the retina in an experimental animal allows for rapid and repeated testing of various surgical tools and maneuvers on a highly vascular tissue *in vivo*. Alternatively, CAM can be excised and pinned to an agarose-lined Petri dish to allow for *in vitro* experimentation. Our group has used both *in vivo* and *in vitro* CAM for comparative testing of the cutting capacity of a novel plasma-mediated dissector called the Pulsed Electron Avalanche Knife (PEAK) versus microsurgical scissors.[22] Techniques that require dissection with minimal bleeding can easily be evaluated for their efficacy on the highly vascularized CAM *in vivo*, since the appearance of bleeding once a vessel is cut or broken offers immediate feedback.

We have also demonstrated the functionality of new coagulation techniques using a specific mode of PEAK on *in vivo* CAM vessels compared to bipolar diathermy (unpublished data, 2002). Chick CAM lends itself to these studies because one can easily visualize blood flow before and after coagulation using either regular surgical illumination (4-degree incidence epi-illumination) or oblique illumination from optical fibers.

Other surgical techniques can be evaluated if the

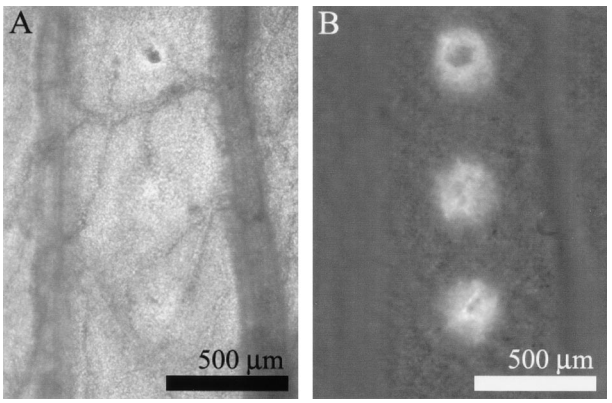

Fig. 3. Use of CAM to visualize damage from surgical instruments. Insults from a Pulsed Electron Avalanche Knife (PEAK) vitreoretinal surgery device were delivered to excised chick CAM using an X-Y-Z micromanipulator in the presence of propidium iodide (a cellular viability marker). Light micrograph (**A**) and corresponding fluorescence image (**B**) showing three insults between two CAM vessels (vessel diameters approximately 150 μm).

ISM that naturally covers the CAM is not removed. Between the ISM and the CAM lies an extensive capillary plexus/blood sinus. Any procedure practiced on the ISM that causes too much mechanical stress to the CAM below will create a noticeable hemorrhage within this plexus/sinus, manifesting itself as a hematoma trapped between the ISM and CAM. This property of the CAM-ISM complex is useful because of its correlative implications for retinal dissection.

Tissue Viability Studies: Simulating Retinal Response to Various Insults

Determining retinal response to various insults is critical for establishing safety data for novel instruments or therapies. One way to collect these data is to expose a suspension of cells to an insult and then measure their response through cell viability markers (e.g., LIVE/DEAD staining).[23–25] This method is not useful, however, if one is interested in tissue viability as a function of distance from the insult. For example, if one were testing a drug or therapy delivered locally to a single point of tissue, one would be interested in the reaction of both the proximally exposed tissue *and* the surrounding distal tissue. In testing a new surgical instrument, it is important to know how tissue adjacent to the instrument responds as a function of distance from the insult and at various instrument settings.

One way to solve this "viability versus distance" problem is to suspend cells in a three-dimensional gel matrix. However, this method carries its own set of disadvantages and difficulties—preparing gels so that cells are uniformly dispersed can be time and labor intensive. Furthermore, the gel matrix may not entirely mimic the physical properties of tissue and therefore may falsely represent the actual effects of the insult of interest. Finally, extracellular cell viability markers (e.g., trypan blue or propidium iodide)[26,27] do not readily diffuse through a gel matrix, thereby preventing optimal staining of compromised cells. Preloading cells with an intracellular marker (e.g., Calcein AM) before casting the matrix is possible, but is more complex to prepare and to image.

One solution to the viability versus distance question is to simply perform tissue viability tests on live animal eyes. This is not ideal because extensive testing in animals is expensive, is time consuming, and raises ethical issues, particularly in preliminary evaluative studies.

We describe CAM as a useful alternative to *in vivo* testing of live animal eyes and believe that it addresses the various issues that accompany viability versus distance studies. For example, CAM can be excised onto an agarose-lined Petri dish that is placed under an inverted fluorescence microscope. Using an X-Y-Z micromanipulator, an insult can be delivered at a precisely measured distance from the tissue and the CAM's response can be monitored after adding a cell viability marker (Figure 3).

Because the CAM is thin and transparent, it is possible to visualize and precisely locate the microsurgical instrument to be evaluated on an inverted microscope despite the CAM's location between the instrument and the microscope objective on the visual axis. CAM's thin and transparent nature also makes it possible to very accurately characterize the lateral extent of damage from an insult. This *in vitro* viability versus distance CAM procedure, which allows for detection of tissue-wide response, is extremely fast and inexpensive. We have confirmed that the tissue viability responses of *in vitro* CAM and *in vivo* rabbit retina to three insults (PEAK, diathermy, and retinal scissors) is similar by using propidium iodide as a marker of cellular viability following each respective insult.

Another advantage of CAM for viability versus distance studies is that partially excised CAM can be used in an *in vivo*/*in vitro* study. A partially excised piece of CAM is pinned to an agarose-lined Petri dish, but a bridge of CAM is maintained back to the egg such that blood continues to flow (at least partially) through the excised portion. This set-up allows the investigator to use the advantages of an *in vitro* set-up (i.e., increased experimental control) while still preserving blood circulation in the tissue, a feature especially important if the insult under consideration is best tested with blood flow intact.

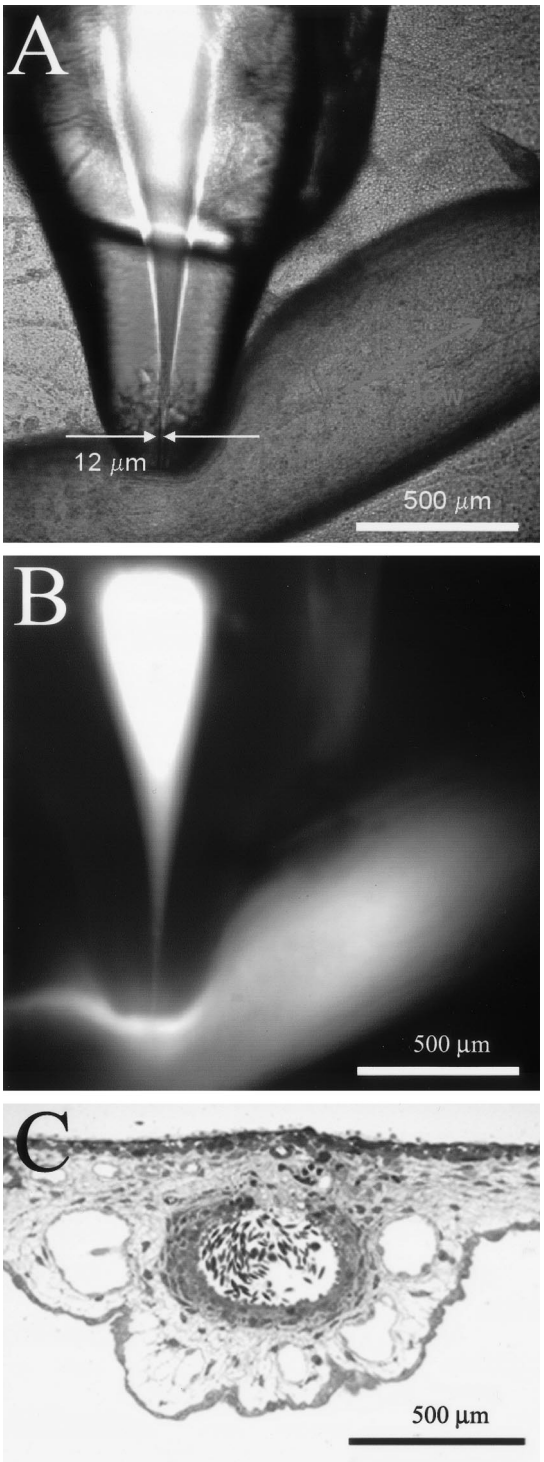

Fig. 4. Use of CAM vessels to test novel therapeutics. Injection of fluorescein into a CAM arteriole using Pulsed Liquid Microjet with a 12 μm nozzle. **A**, Transmission light microscopy of a Microjet nozzle in front of the artery before the injection. **B**, Fluorescence micrograph of the fluid injected through the wall of the blood vessel by the Pulsed Microjet. **C**, Histologic cross-section of the vessel, following intravascular injection.

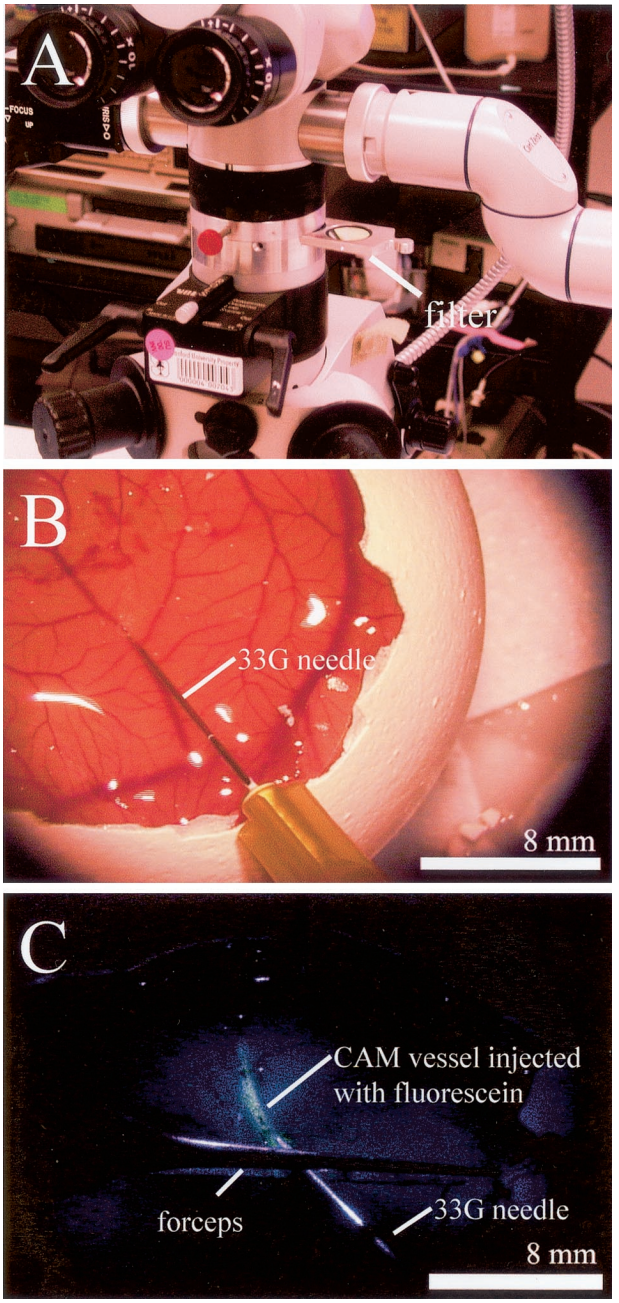

Fig. 5. Cannulation and angiography. **A**, Photograph of surgical microscope equipped with a specially designed removable filter for intraoperative real-time angiography. **B**, CAM vessel that has been cannulated with a 33-gauge needle. **C**, Photograph taken with matched fluorescein excitation (465–490 nm) and barrier filters (525–530 nm) after sodium fluorescein 5% has been injected into a large CAM vessel.

Testing Vessel Cannulation and Injection Techniques

The accessibility of CAM vessels and the ease of imaging these vessels noninvasively makes CAM an ideal tissue for testing vessel cannulation/injection

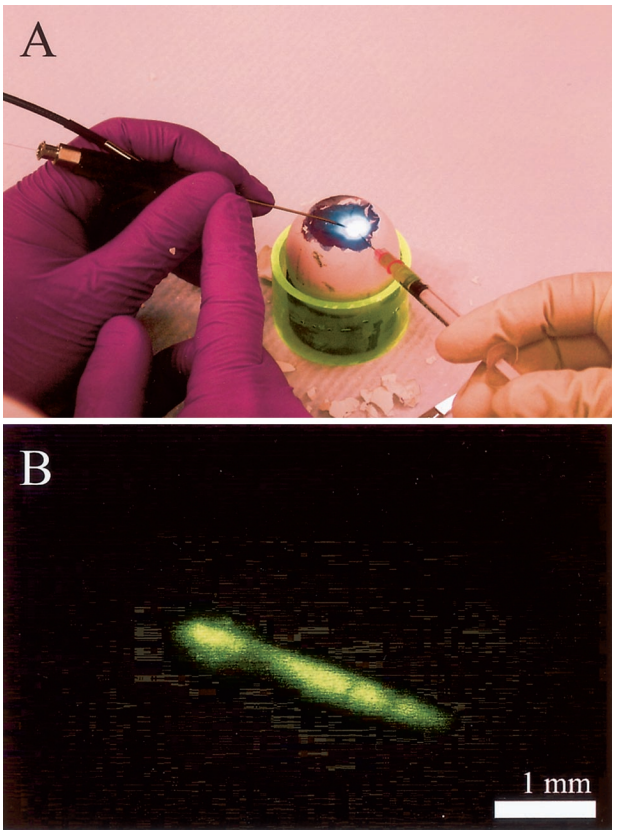

Fig. 6. Endoscopy. A, Photograph showing illumination from an endoscope fitted with fluorescein excitation and barrier filters. **B**, View through the endoscope after a solution of sodium fluorescein 5% has been injected into a large CAM vessel.

techniques. Although CAM vessels vary in diameter, from approximately 1 mm to capillary size, a sizable portion of vessels embedded in the main CAM layer are similar in size to relevant retinal vessels (approximately 40 μm to 350 μm).

For cannulation/injection studies on *in vivo* CAM, observation of individual red blood cell movement and direction of blood flow is easily achieved using a surgical microscope and fiber optics. Distinguishing arteries from veins is possible by observing whether flow in a vessel is pulsatile and ligation is facile when a particular procedure or technique requires it. The cannulation/injection studies we have pursued include (1) testing of a novel Pulsed Liquid Microjet for delivery of microliter quantities of fibrinolytics directly to the site of green-argon laser induced clots[28–32] and (2) the creation of experimental aneurysms and vessel cannulation with a standard 41-gauge needle for fibrinolytic drug infusion[33] (Figure 4). We have found CAM vessels to be an effective model for evaluating new methods for treating clots in the retinal vasculature.

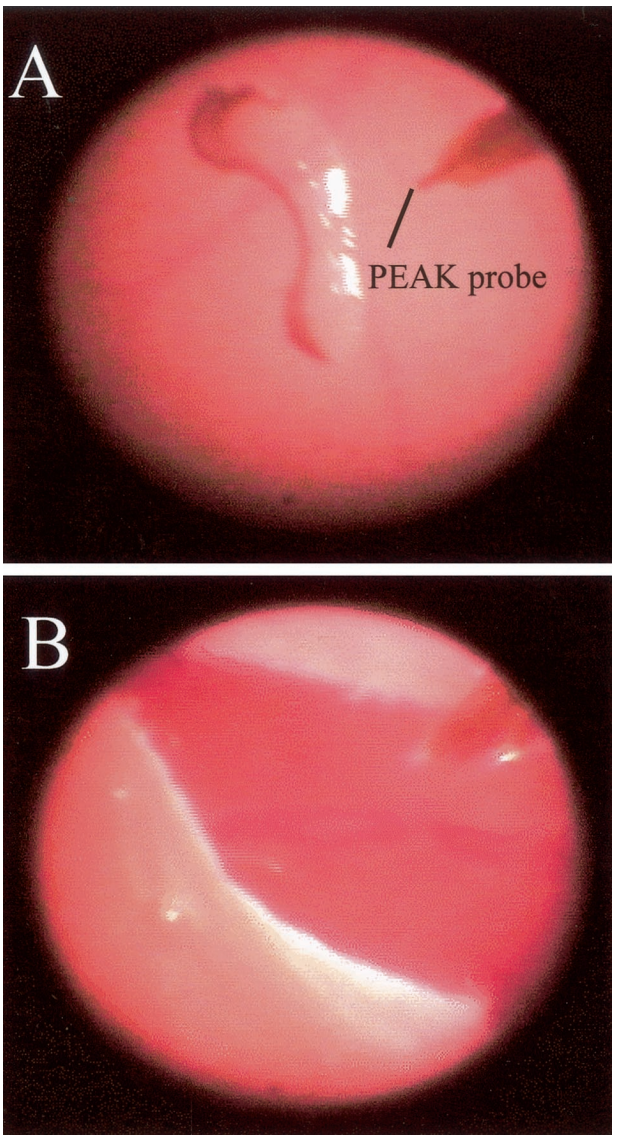

Fig. 7. Endoscopy and instrumentation. **A**, View through a surgical endoscope with a PEAK probe attachment, showing CAM inner shell membrane. **B**, Photograph showing incision made with the PEAK probe using endoscopic visualization.

Angiographic Studies

Although it is sometimes difficult to inject fluorescein into a CAM vessel without a small amount of dye leaking out, it is possible to manually cannulate CAM vessels using small-gauge surgical instrumentation (33–41 G). CAM can be effective in evaluating new dyes and barrier/excitatory filter systems for retinal imaging. It is of value in teaching and practicing manual dexterity to young surgeons or in evaluating new instrumentation and techniques for treatment of retinal vascular disorders. Angiography in CAM vessels may be useful in assessing the effectiveness of

clot formation models and experimental clot lysis therapy. CAM angiography is especially convenient when the surgical microscope used for experiments is equipped with a set of fluorescence filters matched to the excitation and emission spectra of fluorescein (Figure 5).

Endoscopic Surgery

Surgery near and beneath the retina with ocular endoscopes is becoming feasible.[34–36] Because novel surgical techniques and instruments must be rigorously evaluated in nonhuman models before patients are operated on, CAM can provide an inexpensive starting point. Furthermore, CAM can be a convenient practice model for physicians wishing to become proficient and comfortable with handling new endoscopic instruments or techniques before attempts are made during human surgery (Figures 6 and 7).

Conclusion

The CAM's low cost, easy accessibility, anatomic features, and physiologic and histologic responses to manipulation and injury make it a potential model of retina for a variety of surgical applications. While ophthalmic researchers have typically used CAM in the past for angiogenesis studies, we propose and describe several new uses for CAM: (1) testing of novel surgical tools and techniques for cutting, ablating, or coagulating retina and its vasculature; (2) testing vessel cannulation and injection techniques; (3) angiographic studies; and (4) endoscopic surgery.

Key words: angiography, chick chorioallantoic membrane, endoscopic surgery, fluorescence microscopy.

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