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Drug testing and delivery techniques for the *in vivo* tumor spheroid based shell-less chorioallantoic membrane model

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Abstract

We have previously published in detail our methodology of developing the novel tumor spheroid-based shell-less chicken embryo Chorioallantoic Membrane (CAM) model, and its applications in biomedical research. In this article, we will present methods of drug delivery to expand the utility of this model, and also to provide researchers with useful techniques to investigate therapeutic efficacy, drug delivery, and selectivity on this model.

Keywords: chicken chorioallantoic membrane, CAM, spheroid, shell-less, biomedical, tumor, drug testing, drug validation

Introduction

In a previous report[1], we introduced and described the protocol of the *in-vivo* tumor spheroid-based shell-less chicken embryo Chorioallantoic Membrane (CAM) model. This novel twist to the traditional CAM model[1] utilizes implanted three-dimensional spheroids on the chorioallantoic membrane of the chicken embryo resulting in vascularized spheroids without the use of exogenous factors.

The traditional shell-based CAM model, while useful for various tumor biology studies, is not optimal for imaging and biomedical engineering investigations due to restricted access to tumor sites and the relative unstable environment of shell, due to the embryo's ability to move freely in the presence of perturbations[1]. Moreover, drug injections and imaging studies can be challenging[3]. By contrast, the shell-less model offers a stable and flat environment, a large experimental area, and a wider field of view useful for imaging and biomedical engineering applications[1].

Transplantation of pre-formed tumor spheroids into the CAM extends the utility of the shell-less CAM model, permitting the study of later stages in tumor development than otherwise possible. In the avascular stage, tumor spheroids manifest similar early growth and morphological dynamics of solid tumors[2]. After avascular tumor spheroids are implanted directly into the CAM, an *in vivo* environment with pre-existing vascular network, angiogenesis is induced endogenously, creating vascularized spheroids without the use of exogenous growth factors or scaffolds to secure and confine the spheroid inside the CAM[1].

The tumor spheroid-based shell-less CAM model however has its limitations. The relatively short experimental window (approximately 10 days after implantation) over which experiments can be conducted due to the fixed embryonic stage of the chicken embryo, limits the model to short term studies. Long-term therapeutic based studies are thus carried out in more suitable *in vivo* models. Despite its limitations, the model is robust and can be used for both qualitative and quantitative studies of tumor growth, angiogenesis, and therapeutic efficacy in a short experimental time frame[2].

In this report, we describe drug delivery techniques to expand the use of this inexpensive and effective model for the rapid testing and validation of therapeutic drugs *in vivo*.

Materials and Methods

Tumor spheroid-based shell-less chorioallantoic membrane model

The preparation of the spheroid-based shell-less chicken embryo chorioallantoic membrane model has been described in full detail a previous publication[1]. Briefly, the contents of 3-day old fertilized Leghorn or Rhode Island lab grade chicken eggs are transferred to a sterile condiment dish under light restricted conditions (fig. 1). The covered dishes are placed in a ventilated static hatching incubator at 38°C and 60% humidity. On day 7 or 8 of embryonic development, tumor spheroids of 1.0 mm in diameter are implanted in the CAM, followed by a

24-hour incubation period of the embryos to allow the implantation area to heal (fig. 2). Successful implantation is observed when the tumor spheroid is engulfed by the CAM (typically 2 days post-implantation), and the tumor spheroid rests in the highly vascularized mesodermal layer of the CAM. At this stage, the model is ready for use in biomedical investigations[1]. Because hatching occurs approximately on day 21 of the embryonic development stage of the chicken embryo, there is a relatively short time window (approximately 10 days post-implantation) over which experiments can be conducted[1].

Vascularized tumor spheroids from a variety of human derived tumor cell lines have been generated using this method including MCF-7 breast cancer, BXPC-3 pancreatic cancer, NB5 neuroblastoma, U-87 and ACBT (ACBT-G., Granger, University of California, Irvine) glioblastoma cell lines[1]. Some tumor types can develop spheroids naturally through self-assembly or mass growth when placed in suspension, while others require mechanical or chemical manipulation. Not all tumor types however can develop stable spheroids (that is, they will not disintegrate when subject to perturbations).

Fig 1; Top view of the shell-less chicken embryo CAM model. A live chicken embryo on day 3 EA and egg contents inside a condiment dish.

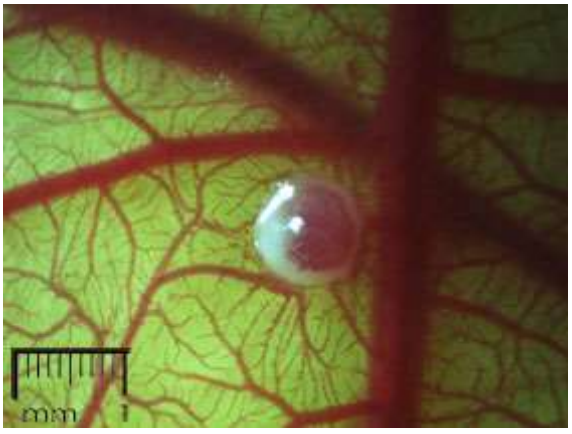

Fig 2 Top view of tumor spheroid and CAM interface – a 1 mm diameter ACBT glioblastoma spheroid inside CAM (mesoderm) on a live chicken embryo on day 14 EA.

Drug and nano-particle delivery techniques

Depending on the goal of the *in vivo* therapeutic study, the therapeutic agent is often administered to the animal model either topically, intraperitoneally, or intravenously. Nanoparticles carrying therapeutic drugs for example, can be introduced into the CAM vascular system either intraperitoneally or intravenously. A drug's therapeutic efficacy, delivery, and selectivity can be assessed with our spheroid-based shell-less CAM model.

The three described delivery techniques were previously tested on our shell-less CAM tumor model using a sterile saline solution 0.9% (NDC 57319-077-08, Phoenix Pharmaceutical Inc) containing blue food coloring for visualization. Here we show the techniques on the CAM only sans tumor implantation. When these techniques are conducted in the spheroid-based shell-less CAM tumor model, the drug delivery and imaging techniques are performed on the CAM 7 days post implantation (day 14 of embryonic development). However, drug delivery studies can be conducted as soon as complete engulfment of the spheroid by the CAM is observed (typically 2 days post-implantation). Sterile instruments, needles, and solutions are used at all times when performing these delivery techniques to minimize the introduction of bacteria and/or mold to the shell-less environment.

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Topical Delivery

In topical administration, the saline solution is applied directly on the CAM/spheroid interface using a pipette, needle or other delivery system of choice (fig. 3a). This method is non-invasive and the CAM is not perturbed. After topical application (fig. 3b), the embryo is returned to the incubator

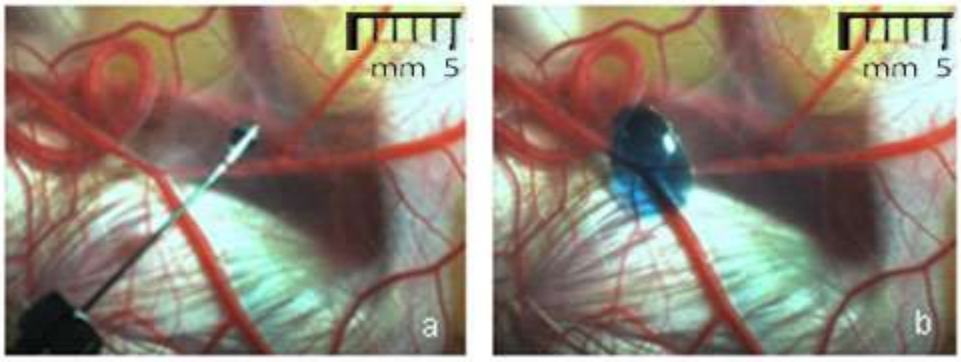

Fig 3 Pictures illustrating the topical application of a blue saline solution on the surface of the CAM.

Intraperitoneal Delivery

In intraperitoneal administration, the saline solution is applied in the abdominal cavity of the chicken embryo. In our CAM/tumor model, the chicken embryos usually rest on the side throughout the extent of the experiment, facilitating the technique (fig. 4a). After locating the thigh of the embryo, the CAM is penetrated using a 1 inch 30G needle attached to a 1cc Syringe without hitting any vessels (fig. 4b). The needle is then inserted in the crevice area between the top of the thigh and bottom of the abdominal cavity using an upward motion towards the abdominal area of the embryo (fig. 4b). One fourth of the needle is inserted, sufficient to cover the bevel of the needle and not to hit vital organs. The saline solution is injected slowly and the needle is retracted after application. The embryo is returned to the incubator. Absorption time is drug dependent and may vary.

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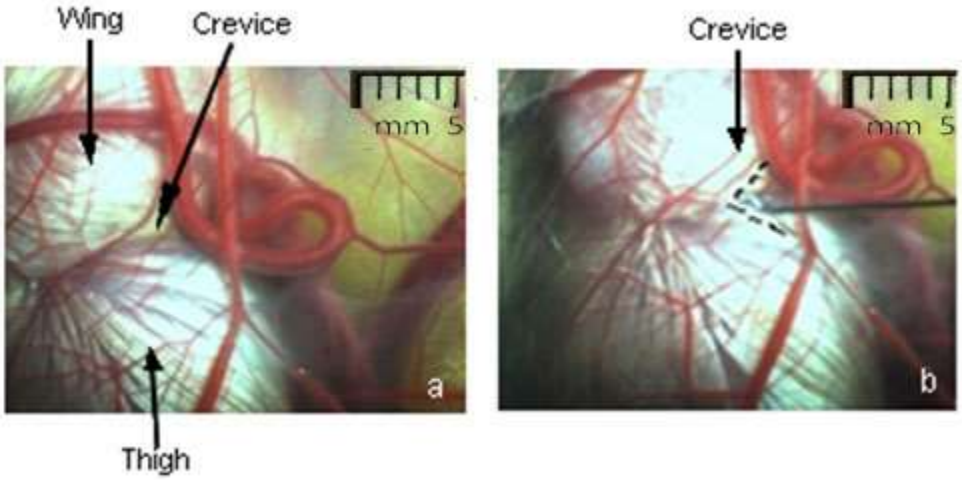

Fig 4 Pictures illustrating the injection of a blue saline solution in the peritoneal area of the embryo. The injection site is in the crevice region between the top of the thigh and bottom of the wing.

Intravenous Delivery

Under a stereomicroscope at 64X magnification, a small vessel of 50 to 100 µm in diameter proximal to the chorionic layer of the CAM (near the surface) is located to minimize hemorrhage (fig. 5a). The diameter of the vessel is chosen so that is slightly larger than the diameter of the needle's bevel.

At a distal area from the CAM/tumor interface, the chorionic epithelium of the CAM and target vessel are held by a pair of tweezers, and a 33G – 36G needle with 30º to 45º angled tip is used to penetrate the chorionic epithelium and target vessel. The needle (reusable fine gauge needle 33x1/2", Popper & Sons, Inc., New Hyde Park, N.Y.) is inserted in the vessel by moving the needle

and the tweezers (holding the vessel) toward each other (fig. 5b). The needle is inserted inside the vessel just far enough for the needle's bevel to completely penetrate.

Once inserted and carefully holding the needle in place (fig. 5c), the blue saline solution is slowly injected inside the vessel (fig. 5d).

After administration of the saline solution, the needle is slowly removed from the vessel, and immediately a disinfected cotton q-tip is applied to the target area with slight pressure to collect the blood (fig. 5e). Continue to apply slight pressure using the q-tip for 10 to 20 seconds until the target vessel collapses and the hemorrhage ceases (fig. 5f). The q-tip is removed, and the embryo is returned to the incubator.

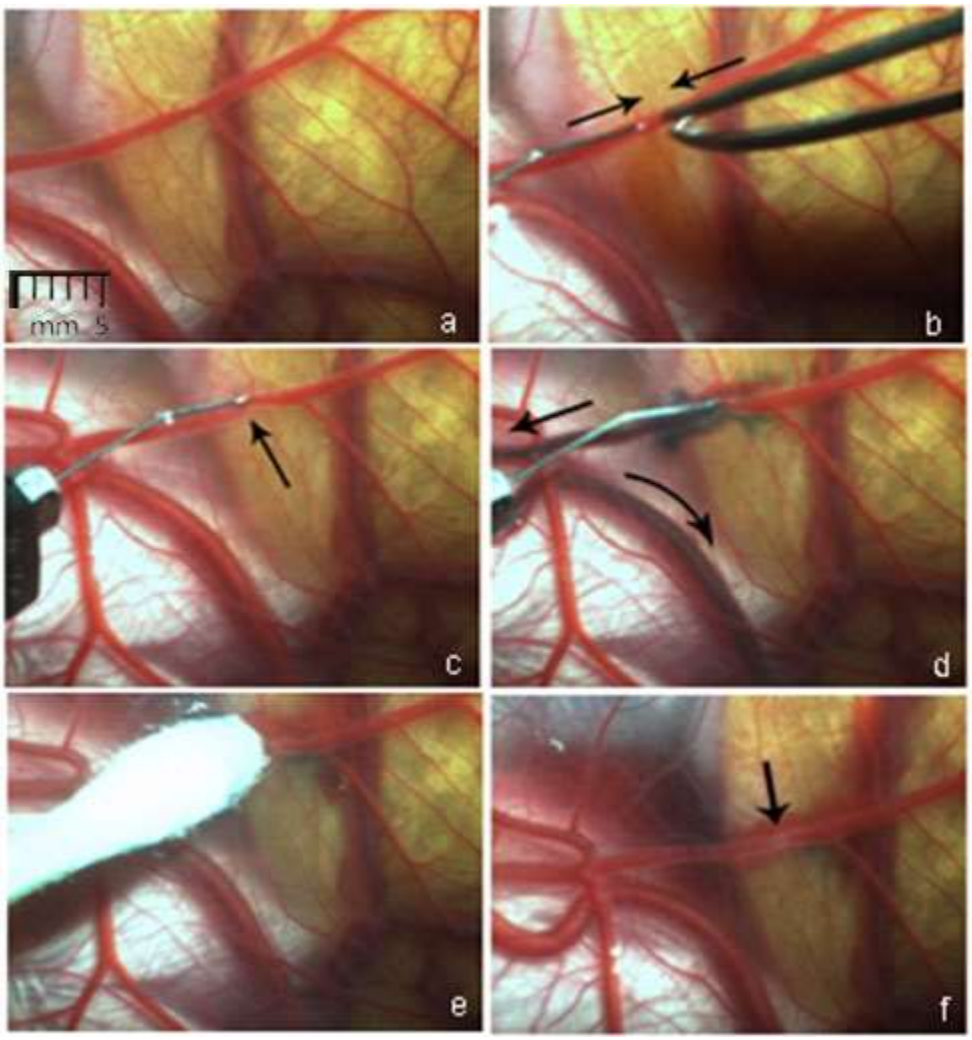

Fig 5 Pictures illustrating the intravenous delivery technique using a 33G reusable needle: a) a small vessel is located; b) holding the chorionic epithelium and target vessel with tweezers, the needle is inserted into the vessel; c) only the bevel of the needle penetrates the vessel; d) a blue saline solution is administered; e) after application, slight pressure is applied to area using a q-tip; f) vessel collapses.

Data Collection Recommendations

During the drug testing experiments, the CAM/tumor interface can be imaged in real time using imaging instruments such as confocal, multi-photon, fluorescence stereomicroscope, and ultrasound imaging devices.

At the completion of the experiment, the CAM/tumor interface can be extracted and processed for further analysis as previously described[1]. For example, subsequent analysis can include histology to assess the drug topography or spatial distribution in the tumor spheroid using imaging agents, drug concentration in the tumor spheroid, and extent of damage to the tumor.

Results and Discussion

While researchers can also test the efficacy of drugs using this CAM/tumor model, the outcome will depend on the delivery mechanism[3,4]. We have introduced the three common delivery techniques ranging from most straightforward (topical) to most challenging (intravenous).

While the topical delivery technique provides fast and direct delivery of the drug to the target site, it doesn't correlate with actual drug delivery methods for cancer treatment in a clinical setting. Two delivery applications that most closely match the clinical setting are intraperitoneal and intravenous delivery. In contrast to topical administration where the exact concentration of the administered drug reaches the target area, intraperitoneal administration results in a slower delivery and lower concentration of the administered drug, because the drug is first absorbed and metabolized by the host system prior to reaching the target area. Moreover, if needle penetration compromises critical organs in the embryo, intraperitoneal administration can lead to embryo lower survival rates. While intravenous administration is the most challenging of the three applications, it provides fast drug delivery since once the drug is injected into a blood vessel, it circulates the closed vascular network of the CAM, and reaches the target tumor faster. It is challenging to secure and penetrate a vessel on the CAM, because the CAM is not rigid and rests on fluid medium. The vessels on the CAM, specifically the venules, are not as rigid since they lack elasticity due to the absence of a smooth muscle network[5-7]. As a result, these vessels are difficult to penetrate as they tend to be slippery, and hemorrhage is almost inevitable after penetration. The hemorrhage can be controlled however, by applying pressure to the punctured area until the vessel collapses. Upon the collapse of the vessel, it has been observed in our experiments that the vascular network on the CAM undergoes rearrangement at the severed area, and creates new network of vessels around the collapsed vessel to re-supply that area with blood flow. This phenomenon has been previously investigated and described in literature[7].

Conclusion

Using the techniques described herein, the spheroid-based shell-less CAM model can be used in short-term studies as an inexpensive transitional model to obtain rapid evaluation and validation of therapeutic drugs and agents *in vivo* without IRB approval, and in many cases without full IACUC review if the chicken embryos are used prior to day 18 of embryonic development (3 days prior to hatching). This model is easily adaptable to a variety of biomedical research applications[2], and it is particularly useful in qualitative and quantitative studies that necessitate monitoring and assessing the efficacy of agents from the avascular to the vascular stages of tumor progression.

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