The chick chorioallantoic membrane as a novel in vivo model for the testing of biomaterials

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Abstract: Current in vivo models for testing biomaterials are time and labor intensive as well as expensive. This article describes a new approach for testing biomaterials in vivo using the chorioallantoic membrane (CAM) of the developing chicken embryo, as an alternative to the traditional mammalian models. Fertilized chicken eggs were incubated for 4 days, at which time a small window was cut in the shell of the egg. After 1 week of incubation, the CAM received several test materials, including the endotoxin LPS, a cotton thread and a Silastic tubing. One day and 1 week later, the tissue response to the test materials was assessed using gross, histological, and scanning electron microscope evaluations. The inflammatory response of the chorioallantoic membrane to biomaterials was fully characterized and found to be similar to that of the mammalian response and was also seen to vary according to test materials. We also found that the structure and geometry of the test materials greatly influenced the incorporation of the samples in the CAM. The similarity of the tissue response of the CAM with the mammalian models, plus the low cost, simplicity, and possibility to continuously visualize the test site through the shell window make this animal model particularly attractive for the rapid in vivo screening of biomaterials. © 2002 Wiley Periodicals, Inc. J Biomed Mater Res 62: 273–282, 2002

Key words: chicken chorioallantoic membrane; biomaterials; animal models; in vivo studies

INTRODUCTION

Currently, tissue responses to biomaterials are evaluated in vivo usually using mammalian models such as mice, rats, and dogs. In addition to ethical issues, mammalian models are both time and labor intensive as well as expensive. Furthermore, implantation of biomaterials in mammalian models does not allow continuous evaluation of the tissue reactions to the implant, but rather requires surgical implantation, animal sacrifice, tissue procurement, processing, and histopathological evaluation. These procedures become even more costly with testing multiple biomaterial configurations and time points. For these reasons, we explored an alternative in vivo approach for the testing of biomaterials. In this article, we describe a novel way of evaluating biomaterials in vivo using the chorioallantoic membrane (CAM) of a developing chicken embryo that allows continuous visualization of the implant site while providing a rapid, simple, and low-cost screening of tissue reactions to biomaterials.

The avian chorioallantoic membrane (CAM) is the outermost extraembryonic membrane lining the non-cellular eggshell membrane (see Fig. 1). The CAM is formed by fusion of the splanchnic mesoderm of the allantois and the somatic mesoderm of the chorion. The fused CAM develops and covers the entire surface of the inner shell membrane of the chicken by day 12 of incubation. The chick will normally hatch at day 21. The CAM serves as a support for the extraembryonic respiratory capillaries, actively transports sodium and chloride from the allantoic sac and calcium from the eggshell into the embryonic vasculature, and forms part of the wall of the allantoic sac, which collects excretory products. Because of low cost, the simplicity of the surgical procedure, and the possibility to continuously observe the test site without disrupting it, the CAM is a common method for studying biological

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processes such as transport,\(^1\) gas exchanges,\(^2\) tumor transplant experiments,\(^3\) toxicity,\(^4\) and more recently angiogenesis.\(^5\)–\(^10\) Typically, an opening is made in the shell to easily access and view the CAM. After having applied drugs, factors, or an implant to the CAM, the window is closed with a transparent tape or a glass slide, thus allowing easy viewing of the test site. This technique has been used for many years; however, it has rarely been used to test biomaterials.\(^11,12\)

The goal of our research was to further characterize the chick embryo CAM as an in vivo model to study the tissue responses to biomaterials using gross, histological and SEM evaluations. Furthermore, in contrast to the previous studies, we also addressed the very important issue of the progressive incorporation of the implants within the CAM after having been placed on top of the membrane. The test materials used in our study included cotton threads, bacterial endotoxin [lipopolysaccharide (LPS)], and Silastic tubings. Our studies demonstrated that the chorioallantoic membrane can be used to accurately evaluate the tissue responses of the CAM to biomaterials over a period of 7–10 days and that the tissue response of the CAM is similar to the tissue response of mammalian models. For example, we observed that a cotton thread (composed of many individual fibers) was more readily incorporated than a Silastic tubing (with a smooth surface), possibly because of the different porosities/topographies of the implants. Both the acute and chronic inflammatory responses can be studied in this model, given the condition that the surface of the implant will readily allow cell adhesion and tissue ingrowth for tissue incorporation to take place. Thus, the CAM model presented in this article can be used as a rapid method of determining tissue reactions to biomaterials and as an intermediate step between the testing of biomaterials in a simpler model (i.e., cells in culture) and a more complex system (i.e., mammalian models).

MATERIALS AND METHODS

Egg windowing

Fertilized chicken eggs were received from the University of Connecticut Poultry Farm (Storrs, CT) and incubated at 37°C with approximately 60% humidity. After 4 days of incubation, the eggs were gently cleaned with a 70% ethanol solution. Using a 5-cc syringe and 18-gauge needle, 2.5 mL of albumen was extracted from the egg. By extracting the albumen, the CAM of the fertilized egg are separated from the top part of the shell, which allows for a small, 1.5-cm\(^2\) window to be cut in the shell of the egg, without damaging the embryonic structures. The window was then sealed using a transparent tape and the egg was placed back in the incubator until day 7 of incubation when test materials were placed on the CAM.

CAM implants

To validate the model for the study of tissue reactions to implants, both the acute and chronic inflammatory responses of the chick embryo chorioallantoic membrane were examined using different implants with different texture and composition and a bacterial endotoxin (LPS). The implants chosen were a cotton thread and a Silastic tubing with dimensions similar to an implantable glucose sensor that we have developed and previously evaluated in rats and dogs.\(^13\) The endotoxin (LPS) was used to induce a severe acute inflammatory response. Acute inflammation was evaluated with these implants and the endotoxin after being placed on top of the CAM for 24 h. The chronic inflammation to the cotton thread and the Silastic tubing was evaluated after 1 week (and also at 11 days for the cotton thread). At these time points, the implants and surrounding tissue were retrieved and examined grossly with a stereoscope and 35-mm camera, sent for histological processing [hematoxylin and eosin (H&E) staining], or inspected by SEM.

Bacterial endotoxins

To induce a severe acute inflammatory response, 100 ng of bacterial LPS endotoxin (Sigma) in 1 μL was placed on top of the CAM of a 7-day-old chick embryo for 24 h. The LPS was mixed with India ink to allow visualization of the distribution of the endotoxin on the CAM. After 1 day, the CAM tissue that received the LPS/ink was retrieved and processed for histology (H&E).
Cotton Thread

To evaluate the CAM inflammatory response to an implant for over a week, 0.5-cm-long cotton threads were used. We had previously used these cotton threads subcutaneously in rats as positive controls of inflammation to implanted glucose sensors.14 After 1 week of incubation (and 4 days after windowing), the samples were placed on top of the CAM of the windowed fertilized chicken eggs. After 24 hours, 1 week and 11 days, the samples were retrieved for gross, histological, or SEM evaluation.

Silastic tubing

Silastic medical grade tubings (0.5 cm long, Dow Corning, 0.031 cm ID × 0.064 cm OD) of dimensions similar to our glucose sensor were sterilized by autoclaving. They were then placed on top of chick CAM after the eggs had been incubated for 1 week (and 4 days after windowing). After 24 h and 1 week, the Silastic tubings with the surrounding CAM were retrieved for gross examination, histology, and SEM.

Evaluation of CAM Responses

Three methods were used to evaluate the tissue response to the endotoxin, the cotton threads, and the Silastic tubings.

Gross evaluation

Before fixation and retrieval, the test materials with the surrounding CAMs were examined under a Zeiss stereo microscope SR at varying magnifications. These gross evaluations were done after 1 day and 1 week of implantation.

Histological evaluation

CAM samples were fixed in 10% formalin, embedded in paraffin, sectioned, and finally stained with H&E for standard histopathological evaluation. Selected histological sections were also stained using trichrome stain to characterize collagen and fibrin deposition within the CAM tissues. Evaluations of CAM were done for various CAM samples retrieved after 1 day and 1 week of implantation. The cotton thread was also examined at 11 days.

SEM evaluation

Topographical and ultrastructural evaluation of the tissue reactions to the thread and Silastic samples were performed using SEM (JEOL) after 1 and 7 days of implantation. The samples were prepared by fixing the implant and surrounding tissue in situ with a 2% glutaraldehyde solution (dissolved in 0.1M cacodylate solution). The samples were critically point dried and processed using standard electron microscopy techniques.

RESULTS

Normal chorioallantoic membrane

A representative histological section (H&E stain) of a normal CAM removed 5 days after windowing the eggs is shown [Fig. 2(A); n = 4] for comparison with the CAMs that received implants for 1 day, 4 days after windowing. The normal CAM is approximately 200 μm thick and is composed of a multilayer epithelium at the air interface (ectoderm), a loose stroma, and a 1-cell layer-thick inner epithelium at the interface with the allantoic sac (endoderm).

![Figure 2](https://www.interscience.wiley.com)
Figure 2(B) shows a representative histological section (H&E stain) of a normal CAM removed 11 days after windowing the eggs for comparison with the CAM that received implants for 7 days, 4 days after windowing \((n = 7)\). Compared with the CAM at 5 days after windowing, the CAM at 11 days after windowing retained the same thickness but with increased cellularity within the stroma. The two epithelia appear to be similar to those shown in Figure 2(A).

As a control, we also evaluated at the same time points, the CAM of chicken eggs that had not been windowed \((n = 7)\). No difference in the CAM was observed between the eggs that were windowed and the eggs that were not windowed (data not shown). This observation suggests that the process of windowing the eggs did not significantly affect the CAM of these eggs.

**CAM reaction to bacterial endotoxin**

The gross morphology of the CAM that received the endotoxin (LPS)/India ink for 1 day, 4 days after windowing, can be seen in Figure 3(A). The LPS was combined with the India ink for ease of visualization. A representative histological section (H&E stain) of the CAM that received the endotoxin is presented in Figure 3(B). Thirteen samples were evaluated. The endotoxin (LPS) induced a strong acute inflammatory reaction after having been deposited on top of the CAM for 24 h. The high density of heterophils and other acute inflammatory cells, such as monocytes, can be seen in the area surrounding the endotoxin/ink. This experiment clearly demonstrated the ability of the CAM to elicit a strong acute inflammatory response similar to mammalian models.

**CAM reaction to cotton thread**

Gross evaluation of cotton threads deposited on top of a CAM for 24 h and 7 days can be seen in Figure 4(A,B), respectively. One day after deposition, the threads were not yet incorporated into the CAM, whereas after 1 week, the threads were incorporated within the CAM with the most intense incorporation occurring at the ends of the threads.

Representative photomicrographs of histological sections (H&E or trichrome stained) of threads that were placed on top of the CAM for 24 h \(N = 10\), 7 days \(N = 5\), and 11 days \(N = 3\) are presented in Figure 4C, D, and E, respectively. When the threads were placed on top of the CAM for 1 day, incorporation had not yet occurred and as a result, the thread is not visible in the histological section [Fig. 4(C)]. However, the location of the thread on the CAM is outlined by a compression of the CAM with a decreased thickness and a mild acute inflammatory response probably caused by the weight of the thread. When the threads were placed on top of the CAM for one week, incorporation of the threads in the CAM had occurred with an associated chronic inflammatory response and
hyperplasia. The histology obtained after tissue retrieval is seen in Figure 4(D). Many leucocytes can be observed around the implant. In addition, giant cells formed around the individual fibers of the cotton threads. Fibroblasts can also be seen congregating around the implant. Remnants of the original ectoderm on which the thread was deposited can be seen at the base of the thread. A new ectoderm grew over
the thread. This chronic inflammatory reaction is similar to what was previously observed when these cotton fibers were implanted subcutaneously in rats. Figure 4(E) demonstrates that when the cotton threads had been placed on top of the CAM for 11 days, the threads became surrounded by fibrosis. The thread can be seen in the lower left corner of the figure. Thread fibers were incorporated within the tissue and the resulting fibrosis is approximately 100 μm across. Many giant cells were also observed around the individual fibers [see Fig. 4(F)]. Other leukocytes can be discerned in the top right hand corner of the figure. These cells are approximately 10 μm in diameter and are likely macrophages. This slide was stained with trichrome for ease in visualization of the collagen and fibrin fibers formed. SEM photographs of the cotton threads incorporated in the CAM were also taken but did not provide any additional information because the threads were totally incorporated within the CAM after 7 days. These studies demonstrated that inflammation to an implant in the CAM progresses from acute to chronic with associated fibrosis, in a manner similar to the inflammatory response of mammalian models.

CAM reaction to silastic tubing

Gross morphology of Silastic tubings deposited on top of a CAM for 24 h and 7 days can be seen in Figure 5, (A,B), respectively. One day after deposition, the tubings were not yet incorporated into the CAM, whereas after 1 week, the tubings were partially incorporated within the CAM.

Representative photomicrographs of histological sections (H&E) of Silastic tubings that were placed on top of the CAM for 24 h (N = 11) and 7 days (N = 11) are presented in Figure 5(C,D), respectively. When the tubings were placed on top of the CAM for 1 day, incorporation had not yet occurred and as a result, the tubing is not visible in the histological section [Fig. 5(C)]. However, like the thread, the location of the tubing on the CAM is outlined by a compression of the CAM shown by a decreased thickness and a mild acute inflammatory response probably caused by the weight of the tubing. When the tubings were placed on top of the CAM for 1 week, their incorporation in the CAM had partially occurred with an associated mild chronic inflammatory response. The histology obtained after tissue retrieval is seen in Figure 5(D). This figure clearly shows the outline formed on the tissue by the tube. In the middle of the figure is the tissue that has grown in the lumen of the tube. Because of its surface composition, the Silastic tubing was not as well incorporated into the tissue as the cotton thread. The smooth surface of the Silastic tubing is less porous than that of the cotton thread, inhibiting cell attachment and growth. Nevertheless, we found that the ends of the Silastic tubing did partially incorporate into the CAM with the tissue growing into the lumen of the tube. In addition, epithelial cells can be seen, migrating around the perimeter of the tube in an attempt to fully incorporate the tubing. A scanning electron micrograph [Fig. 5(E)] taken of the Silastic tubing after 1 week of implantation, clearly shows the CAM tissue growth into the lumen of the tube. The tissue in the lumen was partially retracted because of tissue contraction induced by SEM processing. Interestingly, it can be clearly seen that the tissue failed to grow over the tubing in contrast to the threads that were totally covered by the CAM tissue. Here, the tissue grew only up to mid-height of the tubing. However, the CAM tissue grew inside the lumen of the tubing for a distance of several millimeters as shown in Figure 5(F). Figure 5(F) is the gross morphology of a CAM with the partially incorporated tubings harvested from the egg after 1 week and placed on a Petri dish for ease of visualization. The ingrowth of the tissue into the tube can be clearly observed.

DISCUSSION

The chorioallantoic membrane of the developing chick embryo has rarely been used to test biomaterials in vivo. In 1989, Spanel-Borowski11 described the use of the CAM in the implantation and biocompatibility testing of biomaterials used mainly for hemostypica, vascular prosthesis, and temporary skin substitutes. The implants included both biological as well as non-biologic materials. For the biological materials, Spanel-Borowski used cellulose gauze (Tabotamp), a fibrin tissue adhesive (Tissucol), a collagen sponge (Tachotop), or the gelatin sponge (Gelfoam) and observed that each implant induced different amounts of connective tissue and ulceration. It was also observed that inflammatory cells such as heterophils and the formation of giant cells occurred more often in the gelatin sponge than in the collagen sponge. The non-biologic materials, used mainly as vascular prosthesis or as temporary skin substitutes, included expanded polytetrafluorethylene (ePTFE), polyethylene terephthalate, Dacron, and bilaminar polyurethane foam. Spanel-Borowski observed that expanded polytetrafluorethylene induced squamous metaplasia of the chorionic epithelium. Dacron and the polyurethane foam also showed ulceration of the CAM and bleeding into the implant, respectively. In a recent study, Zwadlo-Klarwasser et al.12 used the chick embryo CAM to study the angiogenic response to some biomaterials and observed that the angiogenic CAM response to biomaterials depends on the chemical com-
position and the physical structure of the material. The authors found that materials with a smooth surface, such as PVC, Tecoflex® (a polyurethane), and HEMA-Tecoflex® appear to be anti-angiogenic, mostly because of the positive charge in its surface. They also found that acid components present in PVC06 and PVC36 or the hydrophilic properties connected with the polyurethane Tecoflex and its HEMA modification is more conducive for capillary growth. The angiogenic/anti-angiogenic effect was viewed as an indication of the relationship between protein adsorption and its dependence on the chemical structure of a biomaterial. Angiogenesis was found to be more readily induced by rough materials, such as filter paper, and a collagen/elastin membrane.\textsuperscript{12}

Although these two articles provided some information on the tissue response to some specific biomaterials, a more detailed study was needed in order to prove that the CAM model can be truly useful to evaluate the tissue response to biomaterials. Specifically, we wanted to show that the tissue response of the CAM is similar to the tissue response of mammal.
lian models such as rats and dogs without the limitations of these models (i.e., cost, need for surgery, time and labor intensive, impossibility to continuously visualize the test sites; see Table I). We also wanted to characterize the incorporation of the test materials in the CAM because, with this model, the test materials are usually placed on top of the CAM instead of being surgically implanted.

Our studies show that the chick embryo CAM can be used to study the tissue response to biomaterials. The inflammatory response of the chorioallantoic membrane to a biomaterial was found to be similar to that of the mammalian response and was also seen to vary according to implants. For example, our studies demonstrated that, depending on the stimulus, the CAM can exhibit a mild (e.g., cotton threads and Silastic tubings) or very severe (e.g., endotoxins) acute inflammatory response. The chronic inflammatory response of the CAM to Silastic tubings and cotton threads was also found to be similar to the response of mammalian models.

This result was not totally unexpected because the tissue responses (i.e., vasopermeability, acute and chronic inflammation, granulation tissue formation, and fibrosis) of mammals and the chick embryo CAM to injury (e.g., bacteria, tumor) are known to be similar. The cells involved in the process of inflammation differ only slightly in the avian model, although their functions are quite similar. Nonlymphoid avian leukocytes include eosinophils, mast cells, basophils, thrombocytes (functional analog to platelets), monocytes, heterophils (functional analog to neutrophils), and macrophages. As in mammalian immunology, thrombocytes, heterophils, monocytes, and macrophages are the major effector cells of nonspecific immunity, namely those cells that are involved in the inflammatory response. The functionally equivalent avian leukocyte to the mammalian neutrophil is the heterophil. Like the neutrophil, it is the characteristic cell of the acute inflammatory response. Experiments performed using artificial irritants and infectious agents have proven the predominance of heterophils in the acute phase of inflammation. Our studies using biomaterials also demonstrated the predominance of heterophils in the early stage of inflammation. As in the mammalian inflammatory process, the initial stage of avian inflammation involves the recognition of the inciting agent, which leads to the production of an array of local inflammatory mediators, such as IL-1. These locally produced chemotactic substances trigger the migration of heterophils and monocytes to adhere to the endothelial cells in the venules and migrate from the bloodstream along increasing concentrations of the chemotactic stimuli, such as IL-8 and IL-1. Heterophils and monocytes begin to infiltrate the tissue in the first 6 to 12 h of the inflammatory response, with macrophages and lymphocytes present at 48 h and giant cell formation at 72 h. In the mammalian model, CAM macrophages are the precursors of macrophages. As they move into the tissues, from the peripheral blood, they undergo an increase in size, an increase in Golgi and polyribosomes, and have an increased hydrolytic enzyme content.

The macrophage will phagocytose any remnants that the disintegrating heterophils may leave and after several days form a syncytium around the area of immunogen. Together with fibroblasts and fibrin, macrophages wall off the site to form a granuloma. Our studies also demonstrated that fibrosis

| TABLE I | Comparison Between the Chick Embryo CAM and Mammalian Models for Testing Biomaterials |
|-----------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| **Chick Embryo CAM Model** | **Mammalian Models** |
| Low cost (eggs, time, labor, etc.) | Moderate to high cost (rats-dogs, per diem, time, labor, etc.) |
| Complete in vivo environment | Complete in vivo environment |
| Very simple surgery to place implants | More complex surgery to place implants (i.e., requires anesthesia, operating room) |
| Allows direct, continuous visualization of implant site through window | Does not allow visualization of implant site |
| Animals do not need to be restrained | Animals often have to be restrained |
| Maximum implantation time of about 2 weeks | Implantation time can be several years |
| Allows evaluation of both acute and chronic inflammation (up to 2 weeks) | Allows evaluation of both acute and chronic inflammation |
| Allows evaluation of granulation tissue in response to injury, including neovascularization. | Allows evaluation of granulation tissue in response to injury, including neovascularization. |
| Fibrosis seen in response to tissue injury and implants. | Fibrosis seen in response to tissue injury and implants. |
| Tissue and blood sampling is easily done (i.e., through window) but amount is limited | Tissue and blood samples are available in larger quantity but require more invasive procedures |
| Biology/physiology of this model is well known (e.g., been used for toxicity, tumor, angiogenesis studies) | Biology/physiology of this model is well known |
| Cost-effective model for utilization of expensive and limited reagents (e.g., cytokines, antibodies) | Cost-ineffective model for utilization of expensive and limited reagents (e.g., cytokines, antibodies) |
and formation of giant cells associated with biomaterials occurs in the CAM model [see Fig. 4(E,F)].

In addition to demonstrating that the tissue response of the CAM to a biomaterial is similar to the tissue response of mammals, we also studied the incorporation of the test materials within the CAM tissue. In contrast to mammalian models, the test materials can not readily be implanted into the CAM but are rather placed on top of the membrane where they slowly become incorporated within the tissue. Our studies indicate that the structure (i.e., porosity, smoothness, geometry) of the materials strongly affects the incorporation. This observation indicates that the CAM model will be most useful with materials that have a structure that will promote incorporation. Clearly, smooth and tall materials that do not promote easy cell adhesion and tissue ingrowth cannot be easily tested using this model unless they are modified. Alternatively, this model could be very useful to evaluate surface modification of materials that are designed to improve cell adhesion and tissue ingrowth.

These studies show that the CAM can serve as an appropriate tool for the screening of biomaterials and their biocompatibility. The CAM model can also be used to study the utilization of tissue response modifiers, such as anti-inflammatory drugs and angiogenic factors, such as human recombinant TNF-α to improve the biocompatibility of implants. TNF-α has been shown to have angiogenic activity on avian cells in the developing CAM. The CAM of the domestic chicken (Gallus gallus) also exhibits more desirable properties for the testing of biomaterials over other CAM models, such as reptiles or even of other avian species. The vascular density of the CAM of a snapping turtle has been reported to be significantly less than that of the chicken. Likewise, the CAM of a developing quail embryo has also been reported to have a slightly lower vascular density. Maintaining an implant area well vascularized is often desirable and so greater vascular density is advantageous to the function and lifetime of the implant.

The in vivo visualization of implants is also highly desirable. Current mammalian models do not allow easy visualization of the implant site but rather requires sacrifice of the animal every time the implant site needs to be assessed. A major advantage of the CAM model is that the egg window allows for visual inspection of the implant as well as easy application of treatments (e.g., drugs, growth factors) to the test site. Perhaps the only disadvantage of this model is that the test materials can be put into the system only for a limited amount of time. The chicken embryo will hatch after 21 days of incubation. Because we need to window the eggs and wait for the full development of the CAM, the time for the implant is approximately 7–10 days. Although we showed this to be enough time for both the acute and chronic response of the tissue, this model is not suitable for long-term studies when other factors (e.g., degradation, mineralization) play a role. However, we believe that the advantages of this animal model clearly outweigh the above disadvantage.

**CONCLUSIONS**

The chick CAM model allows for rapid, simple and low cost screening of tissue reactions to biomaterials. The CAM model is a true in vivo system that can be used as an intermediate step between a cell culture and a more complex mammalian model. The CAM can be used for the evaluation of both acute and chronic inflammatory responses to biomaterials. In addition, the CAM model presents the ability to continuously visualize the implant site without having to sacrifice the test animal. The CAM model may also be advantageous in the testing, screening and modifications of biosensors. Preliminary work in our laboratory has shown promise to this goal.

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