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Development of an *in vivo* model for the study of photodynamic therapy and anti-angiogenic treatments

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ABSTRACT

An *in vivo* model has been developed for the study of photodynamic therapy (PDT) and anti-angiogenic treatments. Significant damage to the vasculature of the chick chorioallantoic membrane (CAM) was observed immediately following PDT with 5-aminolevulinic acid (ALA). Multicell human glioma spheroids were placed on the CAM at day five of embryonic development, however, neovascularization was not observed.

Keywords: Chick chorioallantoic membrane, photodynamic therapy, 5- aminolevulinic acid, glioma spheroids, angiogenesis

1. INTRODUCTION

The CAM is an ideal system for the study of PDT-induced vascular damage¹⁻⁶, growth and neovascularization of tumor nodules and tumor cell suspensions⁷⁻¹², and for the assessment of angiogenic activity^{13,14}. The CAM is an extraembryonic membrane which serves as a gas exchange surface and its function is supported by an extensive capillary network¹⁵. Since the CAM is a transparent membrane, it is possible to observe individual blood vessels and to measure structural changes in the vasculature following various therapies. The CAM is formed at an embryo age (EA) of between 4 and 5 days (EA 4-5); angiogenesis is typically complete at EA 9. Although the chick can hatch as late as EA 20, observation is commonly terminated at EA 17 when the chick immunological system becomes functional¹⁶.

The CAM system is a simple, inexpensive alternative to animal models commonly employed for *in vivo* studies of PDT-induced vascular effects. Unlike animals, the CAM is transparent and light penetration is not a limiting factor. As a result, light activation is not limited to the red region of the spectrum. This allows evaluation of a wide range of photosensitizers which may be applied topically or intraperitoneally^{6,12}. Since this *in vivo* system can also be used as a host for tumor cell transplants, it is an attractive alternative to animal studies seeking to optimize PDT dosimetric parameters.

In this study, the development of an *in vivo* system consisting of human glioma spheroids on a shell-less CAM is described. The model will be used to investigate the response of vasculature and spheroid growth to: (1) ALA-mediated PDT, (2) anti-angiogenic treatments, and (3) combined PDT and anti-angiogenic treatments.

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2. MATERIALS AND METHODS

2.1 Cell cultures

Cells from a grade IV glioblastoma multiforme (GBM) cell line (ACBT- G. Granger, University of California, Irvine) were cultured in DMEM (Invitrogen, Carlsbad, CA) with high glucose and supplemented with 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 $\mu\text{g/ml}$), and 10 % heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA). Cells were maintained at 37 °C in a 7.5 % CO₂ incubator. At a density of 70 % confluence, cells were removed from the incubator and left at room temperature for approximately 20 minutes. The resultant cell clusters (consisting of approximately 10 cells) were transferred to a petri dish and grown to tumor spheroids of approximately 1.0 mm diameter. Prior to placement on the CAM, each spheroid was embedded in collagen gel (collagen concentration - 2 mg ml⁻¹). The collagen disk is typically 3 mm in diameter and 2 mm thick.

2.2 Shell-less CAM preparation

Three-day old fertilized White Leghorn chicken eggs (AA Lab Eggs, Inc., Westminster, CA) were disinfected with 70 % alcohol. Under subdued light conditions, the air pocket at the broad apex of the egg was identified. An 18-gauge needle was used to make a hole at the opposite end (narrow apex). The hole was covered with microporous tape. The shell above the air pocket was carefully removed with tweezers. To ensure that the embryo was properly positioned, the egg was oriented with its air pocket in the down position. Removal of the microporous tape from the narrow apex forced the membrane out of the base of the egg. The membrane was then torn with tweezers and the contents of the egg emptied into a petri dish. The dish was sealed with a semi-porous membrane and placed in an incubator (37° C, 60 % humidity) for 2 days. After 2 days (EA 5), the spheroid/collagen gel was placed on the CAM in close proximity (≤ 1 mm) to a capillary bed.

2.3 PDT of the CAM vasculature

ALA (Sigma, St. Louis, MO) was dissolved in water (100 mg ml⁻¹). At EA 10, 20 μl of 2000 $\mu\text{g ml}^{-1}$ ALA solution in PBS was applied topically to an area demarcated by a silicon O-ring (6 mm i.d..) placed on the CAM and over a region with predominantly small yolk vessels. Laser irradiation was performed approximately 2.5 h following ALA application. The CAM was irradiated with 635 nm light (power density = 25 mW cm⁻²) from an argon ion-pumped-dye laser (Coherent, Inc., Santa Clara, CA). Light was coupled into a 200- μm -diameter optical fiber containing a microlens (Miravant Medical Technologies, Santa Barbara, CA) at the output end. The 25 J cm⁻² light fluence chosen for these experiments correspond to an energy incident on the CAM surface inside the ring area (28 mm²) of 7 J. Five eggs were irradiated in these preliminary experiments and damage assessment was performed by visual inspection using a stereomicroscope (Olympus, model SZH). Images were acquired with a digital camera (Olympus DP 10) coupled to the microscope.

3. RESULTS

The CAM vasculature is clearly evident in Figure 1a prior to irradiation. Significant vessel damage is observed immediately following irradiation (Figure 1b). The absence of small vessels in the irradiated field suggests that they have been destroyed. Figures 2a and b show human glioma spheroids on the CAM. No evidence of neovascularization is seen in either figure.

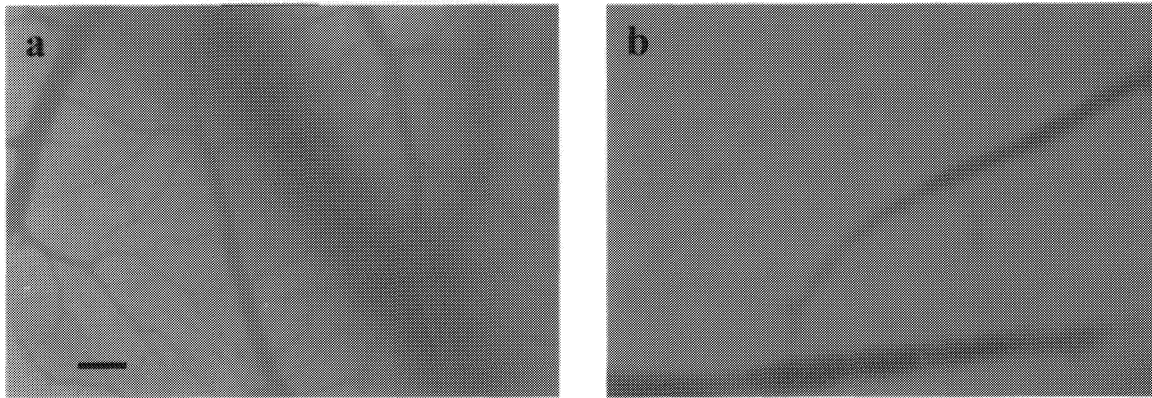


Figure 1. Photographs of the CAM vasculature before (a) and after (b) ALA-mediated PDT. The absence of vessels is evident in (b). The bar represents 0.5 mm.

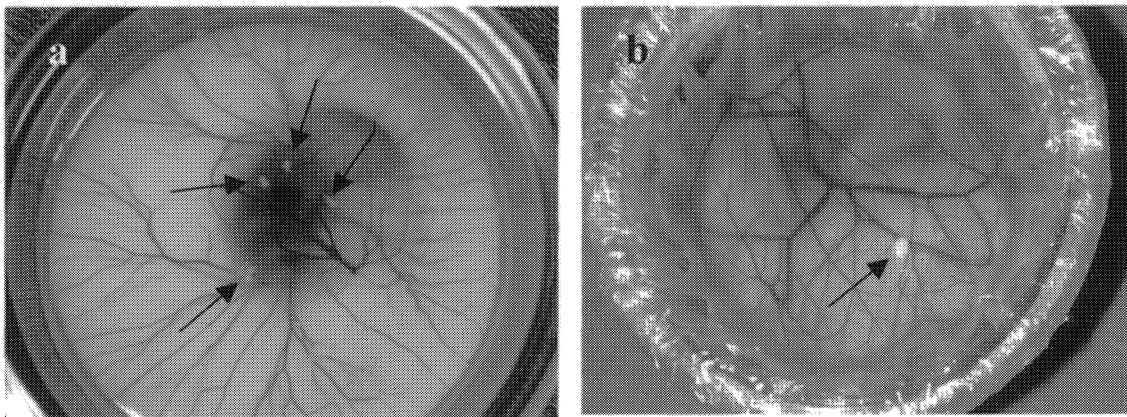


Figure 2. Shell-less CAM at EA 8 (a) and EA 13 (b). Spheroids are indicated by arrows. In (b), the spheroid was embedded in collagen gel prior to placement on the CAM. The chick embryo is clearly visible at the top of Figure 1b.

4. DISCUSSION

Glioblastoma multiforme is a high-grade glioma characterized by a necrotic core and rapid endothelial cell proliferation^{17,18}. There is no satisfactory treatment for this infiltrative neoplasm. Failure of treatment is usually due to local recurrence at the site of surgical resection indicating that a more aggressive local therapy could be of benefit. In 80% of all cases, recurrence is within 2 cm of the resected margin¹⁹. Several studies have shown that photodynamic therapy may prove to be useful in prolonging survival and/or improving quality-of-life in glioma patients^{20,21}. Inhibition of angiogenesis is an alternative strategy for the treatment of these highly vascularized tumors. In fact, GBM would seem to be the prototype of a tumor suitable for anti-angiogenic therapy.

The simple *in vivo* model described in this study was developed to gain a better understanding of the efficacy of treatments that target the tumor vasculature. Although the CAM model is an economical alternative to animal studies, it has numerous drawbacks. Perhaps the most severe of these limitations is the relatively short time window (approximately 10 days) over which studies can be performed. Such short time spans would exclude studies examining the effects of long-term repeat PDT – a procedure that has shown promise in an *in vitro* spheroid model²². Another significant limitation is the nonspecific

inflammatory reactions that may occur following spheroid grafting²³. The resultant vasoproliferative response can impede quantification of the primary response. Nonspecific inflammatory reactions are much less likely when the spheroid is grafted as soon as the CAM begins to develop, while the host's immune system is relatively immature²⁴. The lack of an inflammatory response in this study is likely due to the fact that spheroids were placed on the CAM at a very early stage of embryonic development (EA 5). There are two other important limitations to the CAM assay. First, it is often difficult to distinguish between real neovascularization and falsely increased vascular density that often occurs following rearrangement of existing vessels. This change in vessel architecture is due to contraction of the membrane in response to the spheroid²⁵. Second, in angiogenic studies, timing of the angiogenic response is critical²³. In many studies, angiogenesis is evaluated after 24 h when there is no angiogenesis, only vasodilation. Measurements of vessel density are in reality measurements of visible vessel density, and vasodilation and neovascularization are not readily distinguishable²³.

The shell-less CAM model developed in this laboratory is similar to the system developed by Auerbach et al²⁶. The model has the advantage of providing ready access to the embryo and its membrane for tumor grafting, for introduction of various agents, for surgical manipulations, and for observation of the vasculature. Many spheroids can be placed on each CAM and repeated observation is possible without the problem of trying to locate the spheroids through a small window. The primary drawback associated with the shell-less CAM is the reduction in survival – only 60 – 70 % of chick embryos survive past day 12.

Prior to placement on the CAM, glioma spheroids are embedded in a collagen gel. A similar system using a gelatin sponge has been described by Ribatti et al²⁷. The collagen serves two purposes. First, it anchors the spheroid to the CAM. In the absence of collagen, spheroids show significant displacement from their original position. This is due to the pulsatile motion of the CAM vessels. Second, the collagen acts as a trap to confine test factors (e.g. anti-angiogenic agents) to the site of administration.

The observation that ALA-PDT is capable of causing significant vascular damage in the chick CAM (Figure 1b) is in agreement with the findings of previous studies^{5,6}. The present model was developed in the hope of gaining further insight into the effects of PDT (and anti-angiogenic agents) on spheroid-induced neovasculation. However, to date, neovascularization of implanted glioma spheroids has not been observed in this system. Although angiogenesis is commonly observed in CAMs following implantation of tumor grafts with pre-existing blood vessels^{7,28}, neovascularization of tumor cell suspensions is difficult to achieve unless the cells have been engineered to overexpress angiogenic factors such as vascular endothelial growth factor (VEGF) or fibroblast growth factor-2 (FGF-2)²⁹. Due to the size of the implanted spheroids (1 mm dia.), it is assumed that a significant hypoxic core exists. This is a reasonable assumption since oxygen diffusion is typically limited to between 100 and 200 μm . This is important since it has been shown that the expression of various angiogenic factors, such as VEGF, increases in response to tissue hypoxia^{30,31}. Although spheroids were not assayed for VEGF, its absence would be somewhat surprising given the findings of previous studies showing dramatic upregulation of VEGF expression in GBM cells obtained from patient biopsies³². It should be noted that angiogenesis is a complex process involving not only growth factors such as VEGF and FGF-2, but also matrix degrading enzymes that facilitate endothelial cell migration across the extracellular matrix. Although human neuroblastoma cell lines have been shown to secrete two such factors (matrix metalloproteinase-2 and -9)³³, the ability of GBM cells to produce these enzymes is unknown.

5. CONCLUSIONS

In this study it was shown that ALA-mediated PDT is capable of producing significant damage to CAM vessels. The inability of human glioma spheroids to induce angiogenesis is currently being investigated, primarily by assaying for growth factors such as VEGF and FGF-2.

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