Development of a brain tumor model for investigating the effects of photodynamic and anti-angiogenic therapies

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ABSTRACT

An *in vivo* shell-less chick chorioallantoic membrane (CAM) brain tumor model has been developed to investigate the effects of photodynamic therapy (PDT) and anti-antiogenic treatments. Multicellular human glioma spheroids were placed on the CAM at day 7 of embryonic development. Angiogenesis was observed four days post implantation. Significant damage to the CAM vasculature was observed immediately following 5-aminolevulinic acid (ALA) mediated PDT.

Keywords: Photodynamic therapy, 5-aminolevulinic acid, glioma spheroids, chick chorioallantoic membrane, angiogenesis, ACBT cells

1. INTRODUCTION

ALA-mediated PDT has many features that make it a promising adjuvant therapy for the treatment of brain tumors. Introduction of ALA in biological systems leads to the overproduction of the natural occurring photosensitizer protoporphyrin IX (PpIX), through the heme biosynthetic pathway[1]. Favorable tumor-to-normal tissue localization, rapid clearance from cutaneous tissues, oral and/or topical administration, and the possibility of repeated treatment make ALA an attractive compound for PDT[2]. Additionally, the multi-targeting feature of PDT allows tumor growth disruption and angiogenic vascular damage to be accomplished in one treatment.

The CAM system is a simple and attractive alternative to animal models for *in vivo* studies of PDT-induced tumor and vascular effects[3-11]. Unlike animal tissue, the transparency of the CAM allows for optimal imaging and observation of vasculature and tumor response during therapeutic studies. The shell-less CAM system provides a larger working area for better observation, and allows for the placement of more tumor samples required for the study of multiple PDT parameters at one time.
The main drawback of the CAM system is the relatively short time window (approximately 10 days) over which experiments can be conducted. Studies of long-term PDT effects and repeated PDT treatments on angiogenesis and tumor growth are not feasible.

In this study, an *in vivo* shell-less CAM brain tumor model has been developed to study the effects of PDT and anti-angiogenic treatments on vasculature and spheroid growth. The model and preliminary findings are discussed.

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2. MATERIALS AND METHODS

2.1 Cell Cultures

Cell from a grade IV glioblastoma (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 µg/ml), and 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA). Cells were maintained at 37 ^{0}C in a 7.5% CO_2 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, clusters of 1-4 spheroids were embedded in a 1:1 matrigel and collagen I (2 mg ml^{-1} concentration) gel. The collagen/matrigel disk was typically 3 mm in diameter and 2 mm thick. The gel serves to anchor the spheroid on the CAM, and to confine test factors (e.g. anti-angiogenic agents) to the application site. In prior experiments, spheroids showed significant displacement from the original application site in the absence of the gel.

2.2 Shell-less CAM preparation

Three-day old fertilized brown Leghorn chicken eggs (AA Lab Eggs, Inc., Westminster, CA) were disinfected with 70% alcohol. Under restricted 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 whole was covered with microporous tape. The shell above the air pocket was carefully removed with flat square-head 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 flat square-head tweezers and the contents of the egg emptied into a deep condiment type dish (Wal-Mart, Inc.). The dish was sealed with a semi-porous membrane and placed in an incubator (37 ^{0}C, 60% humidity) for 4 days. After 4 days (Embryonic Age, EA 7), the collagen/matrigel embedded spheroid cluster was placed on the CAM in close proximity (≤1 mm to a capillary bed).

2.3 PDT on the CAM-spheroid interface

ALA (Sigma, St. Louis, MO) was dissolved in deionized water (100 mg ml^{-1}). A 250 µg/ml ALA solution in 1X CO_2 independent culture media (Invitrogen, Carlsbad, CA) was prepared. At EA 11, a silicon O-ring (6 mm diameter) was placed on the CAM and centered on the spheroid clusters. The purpose of the silicon boundary was to confine the ALA to the spheroid clusters. Previous experiments showed that ALA application on the entire CAM resulted in embryo death. 100 µl of the ALA solution was applied topically inside the ring. Laser irradiation was performed approximately 3.0 h following ALA application. The CAM was irradiated with 637 nm light (power density = 25 mW cm^{-2}, energy density = 25 J cm^{-2}, beam spot diameter = 1.2 cm) from a IQ1C20 laser diode module (Power Technology, Inc., Little rock, AR). Light was coupled into a 600-µm-diameter optical fiber (Medlight SA, Switzerland) containing a micro lens at the output end.

2.4 Imaging

Angiogenesis and damage assessment was performed by visual inspection using a stereomicroscope (Olympus, model SZH) at magnifications of 10x, 22x, 44x, and 64x. Images were acquired with a digital camera (Olympus DP 10) coupled to the microscope.

3. RESULTS

Figure 1 illustrates the results of the angiogenesis assessment study employing twenty-two embryos. The CAM microvasculature surrounding the ACBT spheroid cluster in figure 1b is indicative of angiogenesis induction four days post spheroid implantation. These vessels are absent after only one day of culture as seen in figure 1a.

3.1 ACBT spheroids

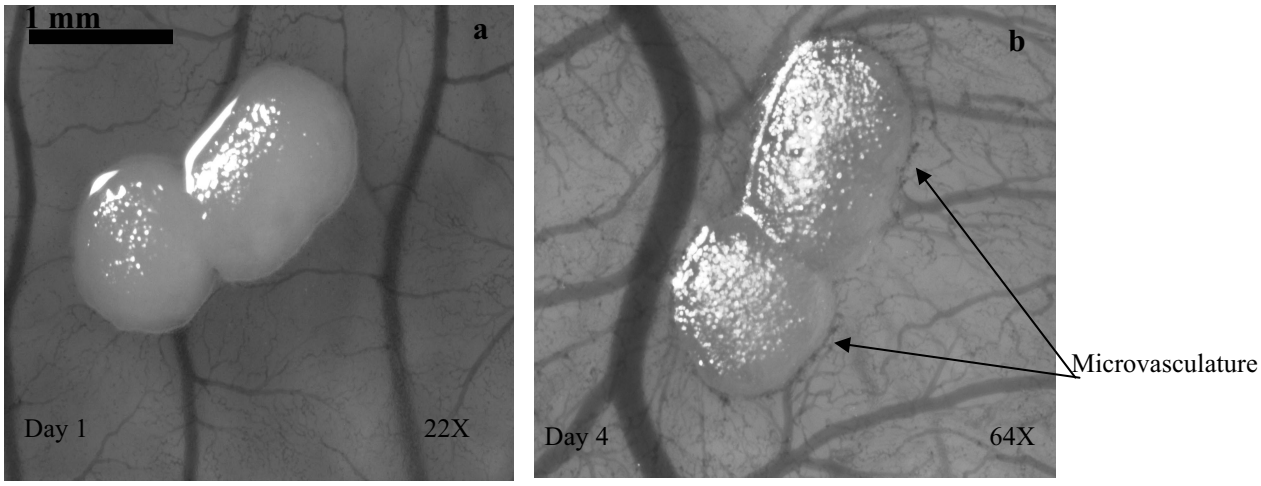

Figure 1. (a) Images of ACBT spheroid cluster one day after implantation at EA 8, and (b) 3 days after implantation at EA 11.

3.2 Controls

Control cultures consisting of normal fibroblasts (Figs. 2a and b) or matrigel alone (Figs. 3a,b) were also evaluated to see what role they might play in the induction of angiogenesis. As can be seen from the figure, the microvasculature network observed surrounding the tumor spheroids (Fig. 1b) is absent in the fibroblast only (Fig. 2a and b) and collagen/matrigel only (Fig. 3a and b) control groups.

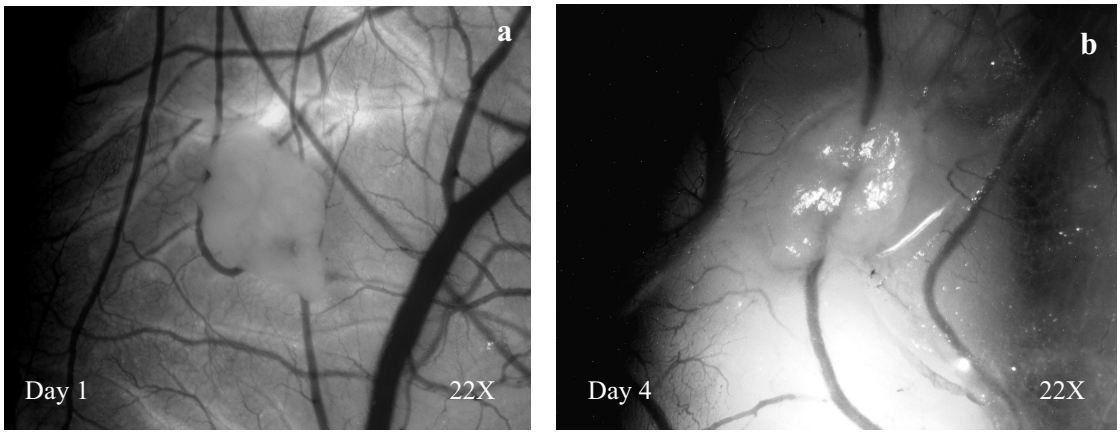

Figure 2. (a) Image of Fibroblast cluster one day after implantation at EA 8, and (b) 3 days after implantation at EA 11.

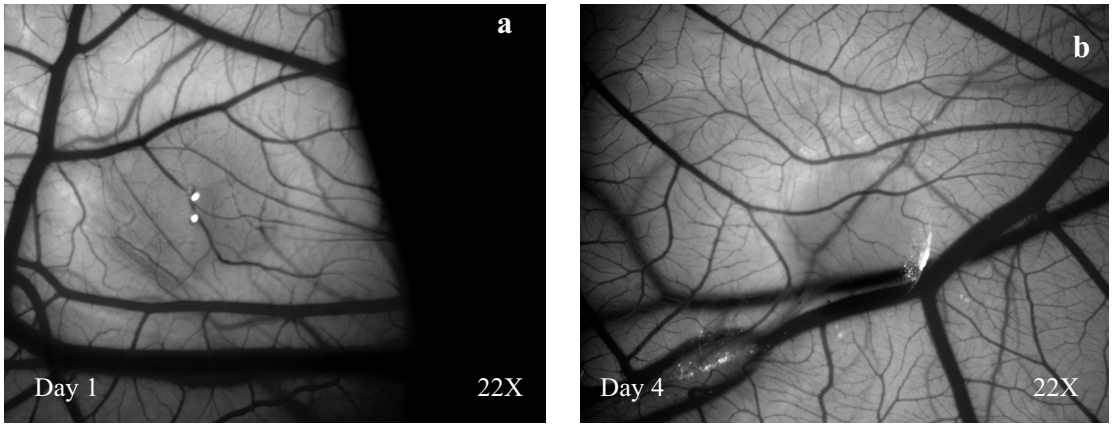

Figure 3. (a) Image of Collagen/Matrigel only drop with green food dye, one day after implantation at EA 8, and (b) 3 days after implantation at EA 11.

3.3 ALA–mediated PDT

Three embryos were irradiated in an ALA-PDT preliminary study. The increased vasculature seen after 4 days of incubation is evident (Fig.4a). Damage to the CAM vasculature and microvasculature network after ALA-mediated PDT is evident in Figure 4b.

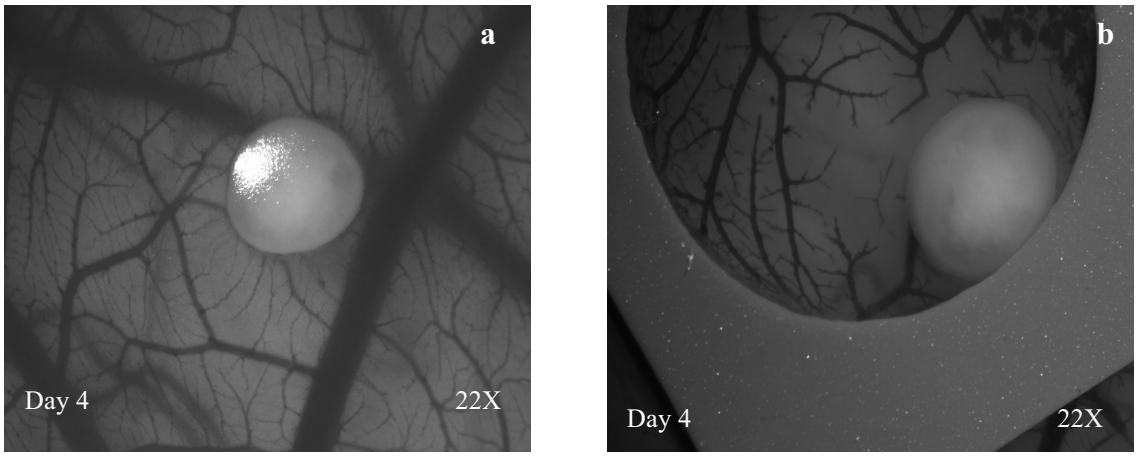

Figure 4. Images of shell-less CAM model interface before (a) and after (b) ALA-mediated PDT.

4. DISCUSSION

Glioblastoma multiforme is a high-grade glioma characterized by a necrotic core and rapid endothelial cell proliferation. Several studies have shown that photodynamic therapy may prove to be useful in prolonging survival and/or improving quality-of-life in glioma patients. 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 present model was developed in the hope of gaining further insight into the effects of PDT (and anti-angiogenic agents) on glioma spheroid-induced neovasculature.

The CAM microvasculature surrounding the ACBT spheroid cluster in Figure 1b is evidence of angiogenesis induction four days post spheroid implantation. This effect was observed without the use of exogenous angiogenic factors such as vascular endothelial growth factor (VEGF) or fibroblast growth factor-2 (FGF-2). Neovascularization of tumor cell suspensions is readily observed in CAM systems with tumor cells engineered to overexpress angiogenic factors[12]. The present shell-less CAM *in vivo* model illustrates that GBM spheroid-induced angiogenesis is possible with non-engineered tumor cells. This form of vascularization allows the study of the tumorigenic behavior of gliomas without the application of exogenous signals that could otherwise interfere with the natural processes of tumor proliferation.
Although fibroblast cells can secrete FGF-2, and the extra cellular matrix is known to contain angiogenesis promoting factors, a similar microvasculature network to that observed in the presence of tumor spheroids (Figure 1b) was absent in the fibroblast-only (Figures 2a-b) and collagen/matrigel only (Figures 3a-b) control groups.

Damage to the CAM vasculature and microvasculature network (Figure 4a) after ALA-mediated PDT is evident in Figure 4b. This shows that PDT is effective in causing vascular damage on the CAM. Previous studies have reported similar findings[13, 14]. Histopathological and anti-angiogenic studies are currently being conducted to further validate the above preliminary findings.

5. CONCLUSION

The *in vivo* shell-less CAM brain tumor model described in this study was developed to gain a better understanding of the angiogenic, proliferative, and invasive processes of Glioblastoma multiforme, and the effect of PDT and anti-angiogenic therapies on these processes. By using this model, we were able to observe GBM spheroid-induced angiogenesis in the absence of exogenous angiogenic factors four days after implantation. Additionally, we were able to show that PDT is effective in causing vascular damage on the CAM. Currently, the CAM model is being used to investigate the invasive activity of GBM tumor spheroids and the effect of PDT on tumor migration.

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