Investigating the effects of combined photodynamic and anti-angiogenic therapies using a three-dimensional *in-vivo* brain tumor system

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ABSTRACT

An *in-vivo* tumor model composed of multicellular human glioma spheroids implanted on a shell-less chorioallantoic membrane (CAM), has been developed. Following removal of a portion of the ectodermal epithelium layer of the CAM, human glioma spheroids were implanted on day 7 of embryonic development. Tumor invasion, rapid growth and vasculature formation were observed 7 days post implantation. Single tumor cell migration towards blood vessels, angiogenesis and satellite tumor growth were also evident.
The human tumor/CAM model is being used to examine the effects of combined ALA PDT and anti-angiogenic agents. The shell-less CAM is well suited for topical, i.p. and i.v. photosensitizer and/or drug application.

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].

The CAM system is a simple, inexpensive 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. In addition, the shell-less CAM system provides a larger working area for better observation, a stable flat environment for consistent analysis, simulation of the tumor environment, and faster acquisition of experimental results.
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 system has been improved to study the effects of PDT and anti-angiogenic treatments on vasculature and spheroid growth. The system and preliminary findings of angiogenesis induction and acute PDT are discussed.

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2. MATERIALS AND METHODS

2.1 Cell Cultures

Cells 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.

2.2 Shell-less CAM/Tumor system preparation

Three-day old fertilized white 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 hole 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. The dish was sealed with a semi-porous membrane and placed in an incubator (39 ^{0}C, 60% humidity) for 4 days.
After 4 days (Embryonic Age, EA 7), one spheroid of approximately 1.0 mm diameter was implanted on the CAM in close proximity to a large blood vessel. The embryo was incubated for 4 additional days.

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 double deionized water was prepared. PDT was conducted on day 7 (EA 14). 50 µl of ALA solution was injected into the embryo intraperitoneally (i.p.) using a 30 gauge needle[14]. The embryo was placed in the incubator, and PPIX fluorescence was examined using a 366 nm U.V. lamp (model UVL-21, Upland, CA) 30.0 min , 1.0 h, 1.5 h, and 3.0 hr after injection. Optimum PPIX fluorescence was observed at 1.5 h. Laser irradiation was performed approximately 1.5 h following ALA application. The CAM/spheroid interface was irradiated with a 637 nm light (power density = 25 mW cm^{-2}, energy density = 25 J cm^{-2}, beam spot diameter = 1.2 cm, irradiation time = 32 min) 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.
A pinhole of approximately 3 mm in diameter was used to minimize scattering and to focus the PDT treatment on the CAM/spheroid region.

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.

2.5 Histology

Immediately after irradiation, the CAM/spheroid interfaces were fixed with 10% formalin, removed from the CAM, and stored in 10% formalin solution overnight. The samples were processed, embedded in paraffin and cut in 6 µm serial sections. The sections were stained with hematoxylin and eosin.

3. RESULTS

3.1 Angiogenesis

Figure 1 illustrates the results of the angiogenesis assessment study employing thirty embryos. Figures 1a and 1c illustrate the CAM/tumor spheroid interface at the endo layer and epi layers of the CAM respectively (44x). Figures 1b (10x) and 1d (64x) show the histology of the CAM/tumor interface. The CAM completely surrounds the tumor (fig. 1b), and microvasculature is evident inside the ACBT spheroid (fig. 1d) indicative of angiogenesis induction four days post spheroid implantation.

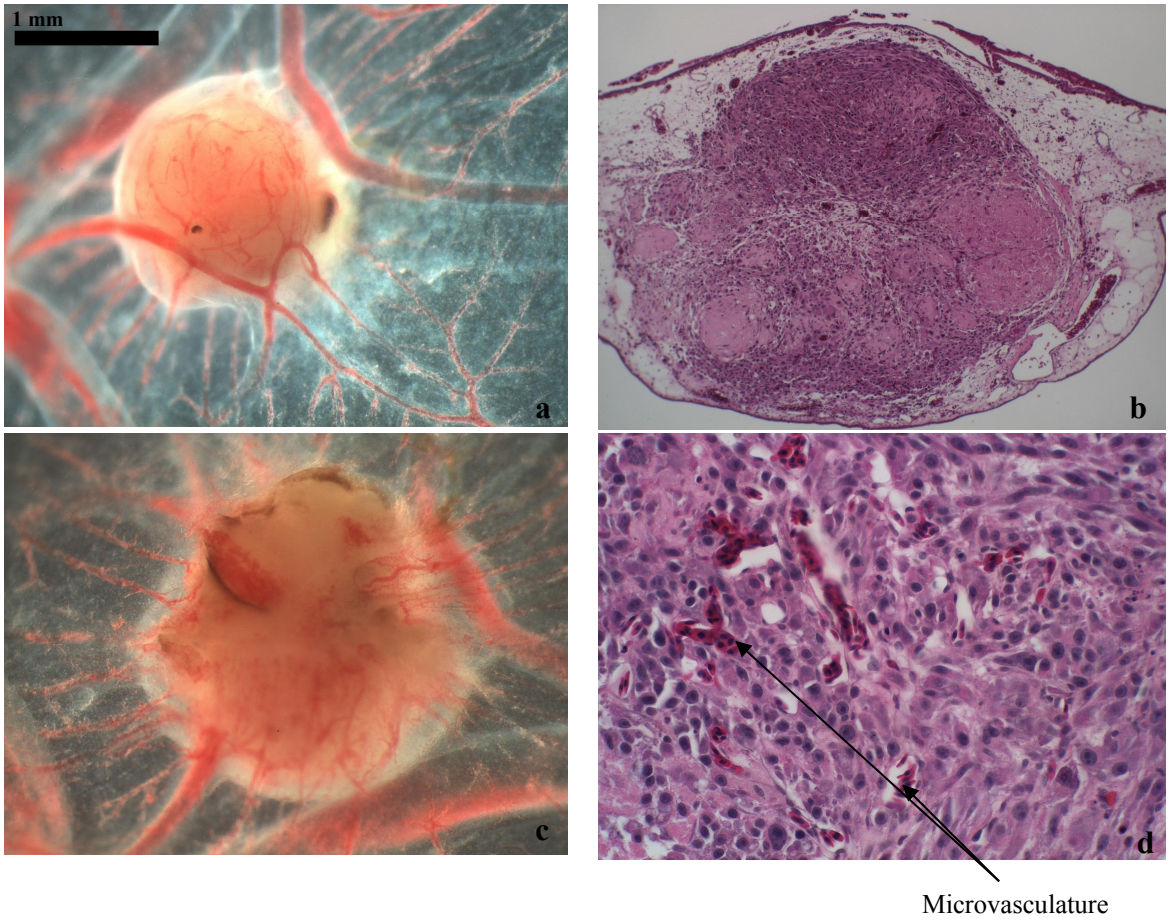

Figure 1. (a,c) Images of ACBT spheroid 7 days after implantation at EA 14, and (b, d) histological sections.

3.2 ALA-mediated PDT

Fifteen embryos were irradiated in an ALA-PDT preliminary study. Immediately after PDT (acute), hemorrhage is observed in the center vasculature network of the spheroid at the endo layer (fig. 2c) and epi layer (fig. 2a) of the CAM. Histological sections show breakdown of the spheroid extracellular matrix network (fig. 2b, d), and occlusion of microvasculature.

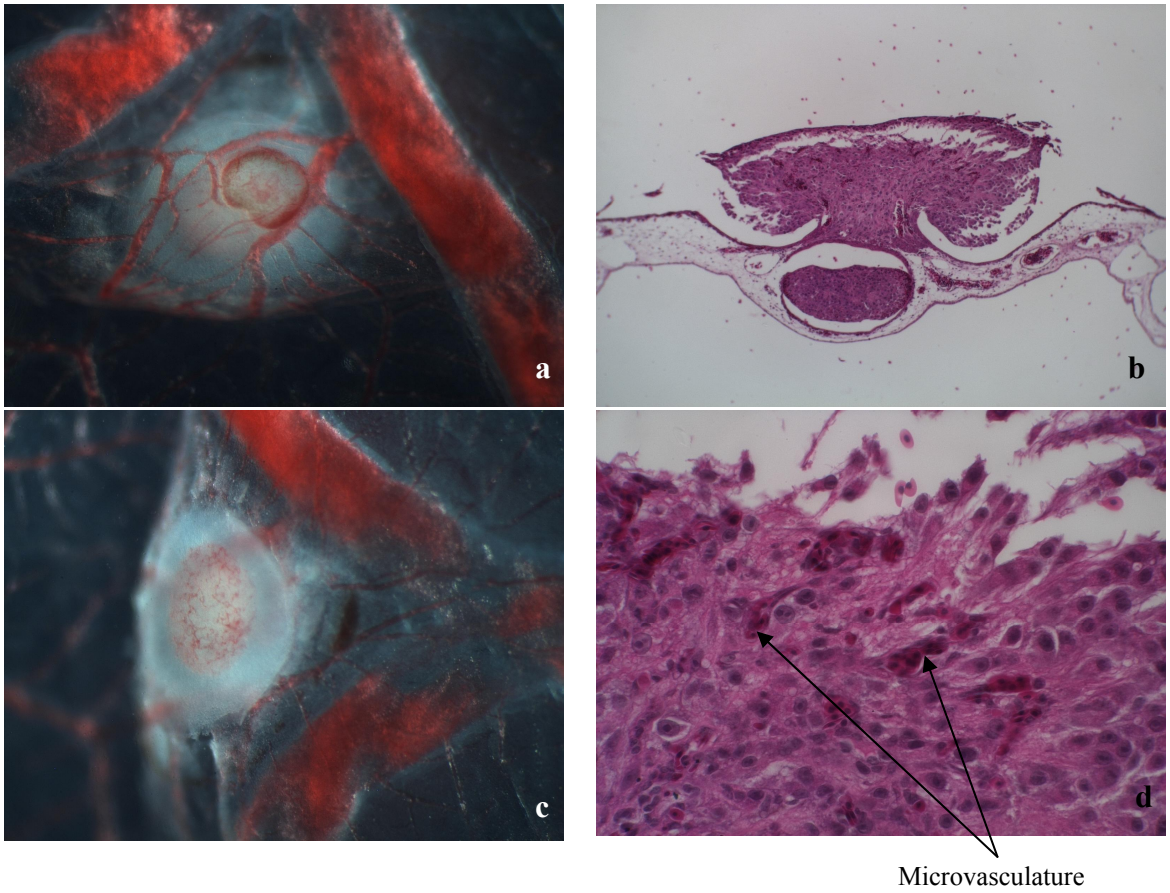

Figure 2. (a,c) Images of ACBT spheroid post irradiation (acute) 7 days after implantation at EA 14 , and (b, d) histological sections. Each CAM/spheroid region was irradiated with a 637 nm light (power density = 25 mW cm^{-2}, energy density = 25 J cm^{-2}) for 32 min.

3.3 Normal and PDT comparison

Damage to the tumor cells, extracellular matrix (ECM) and microvasculature (occluding) after acute ALA-mediated PDT is evident in Figure 3b.

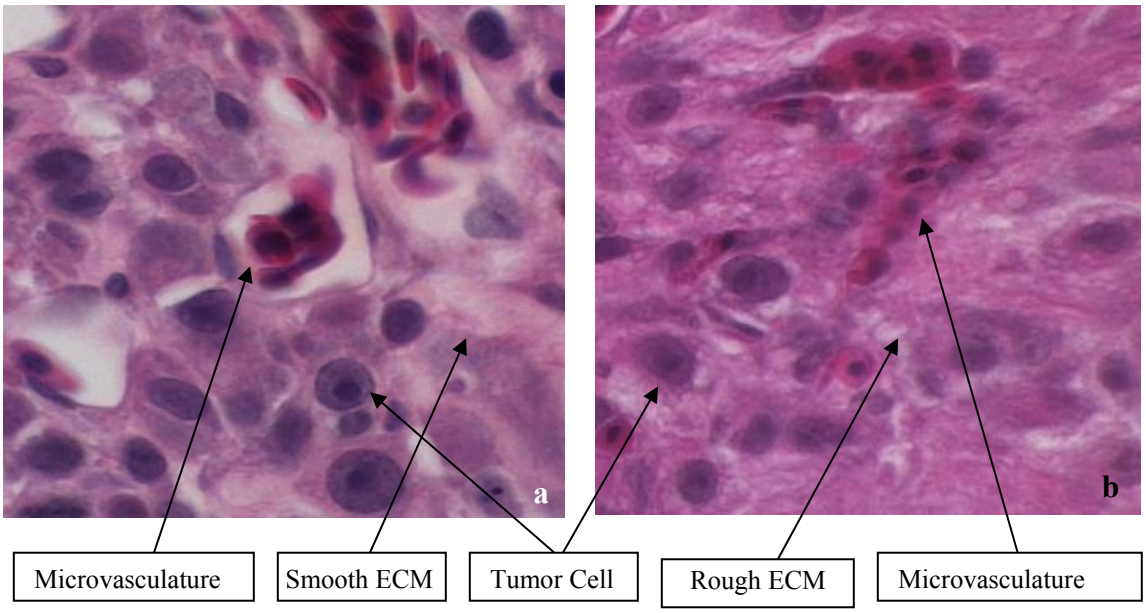

Figure 3. (a) Image of normal spheroid 7 days after implantation at EA 14. (b) Image of spheroid after PDT at EA14.

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 inside the spheroid in Figures 1b and 1d 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 *in vivo* CAM 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.

Damage to the tumor microvasculature network (Figure 3) after ALA-mediated PDT is evident in Figure 3b. Individual blood cells are no longer distinct, and microvasculature lining is no longer visible (figure 3b), compared to normal tumor vasculature observed in figure 3a. The tumor cell shape and nucleus are no longer round. This shows that PDT is effective in causing vascular damage inside the tumor and on the CAM. Previous studies have reported similar findings[13, 14]. Additionally, The extracellular matrix (ECM) of the tumor environment is observed to have a rough characteristic after PDT treatment. Anti-angiogenic and longer term (12 hours, 24 hours, 3 days) PDT effect studies will be conducted to further validate the above preliminary findings.

5. CONCLUSION

The *in vivo* shell-less CAM brain tumor system 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 system, 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 damage to the tumor cells, extracellular matrix network, and tumor vasculature.

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