Original Research Article

Antiretroviral therapy and HIV-associated cancers: Anti-angiogenic effect of efavirenz on chick chorioallantoic membrane

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Abstract

Purpose: To investigate the anti-angiogenic effect of efavirenz in chick chorioallantoic membrane (CAM).
Methods: Fertile eggs of the domestic fowl (Gallus gallus, variant Domesticus) were used as the in vivo vascular test environment. Experimental groups were VEGF, thalidomide (negative control), efavirenz (test drug), control 1 (treated with diluent) and control 2 (no treatment). CAM photographs were taken using a microscope camera. CAM blood vessels were scored as either "present" or "absent".
Results: There was association between treatment drugs and growth of CAM blood vessels ($p < 0.05$) as follows: control 1 – 100 %, control 2 – 67 %, VEGF - 83 %, thalidomide - 17 %, and efavirenz - 0 % (n = 30). Although efavirenz had absolute anti-angiogenicity and presented as a potential anti-cancer drug, there was no significant difference between the anti-angiogenicity of efavirenz (100 %) and thalidomide (83 %) ($F = 1.000$, $p = 0.341$).
Conclusion: Being a first-line drug in both HAART and combination treatment of HIV-1, efavirenz may be responsible for spontaneous resolution of secondary cancers in HIV-infected patients on HAART regimen.

Keywords: Efavirenz, HIV, Cancers, Angiogenesis, Chick chorioallantoic membrane

INTRODUCTION

Kaposi sarcoma is the most common cancer seen in patients with acquired immunodeficiency syndrome (AIDS). Many researchers have documented the regression of HIV-associated cancers after antiretroviral therapy (ART) administration [1-5]. This has been particularly reported for lung cancers [6] in relation to the use of "highly active antiretroviral therapy" (HAART). HAART is a drug schedule that involves the use of Efavirenz [7]. HAART induces tumor regression [8] and increases the survival rate among HIV patients with pulmonary Kaposi sarcoma [9]. HAART regime has therefore changed the natural history of HIV/AIDS-related cancers [8]. Moreover, it lengthens the time from onset of treatment to treatment failure [10]. Efavirenz is the most widely used non-nucleoside reverse transcriptase inhibitor (NNRTI) [11].

Vascular endothelial growth factor (VEGF) is a specific and potent mitogen for endothelial cells and its roles in development and pathological angiogenesis have been documented [12,13]. It promotes cancer proliferation and metastatic spread by stimulating growth of new vascular networks [14]. The current interest in thalidomide

lies in its anti-angiogenic properties [15-17]; it antagonizes angiogenic factors such as VEGF [18] and Tumor necrosis factor alpha (TNF-a) [19,20]. Efavirenz is the most widely used NNRTI, and is a first line drug in the management of HIV in both adults and children. In combination with other anti-retroviral drugs [21] it suppresses viral load, thereby increasing CD4 count. Nevertheless, Efavirenz has not been adequately studied, even in patients with advanced HIV disease (patients with CD4 counts < 50 cells/mm[3)] [22].

EXPERIMENTAL

Ethics approval

The work was carried out at the University of the Witwatersrand Medical School, under Ethics clearance certificate no. 2008/7/1, and also followed the guidelines in the NIH publication no. 85-23, revised 1985 ("Principles of Laboratory Animal Care") [23].

Animals

Thirty (30) freshly laid, fertile eggs of the New Hampshire breed of chicken (*Gallus gallus* variant *domesticus*) and of the same clutches were incubated on the 3rd day of development (Hamburger and Hamilton's stage 21) and used as *in vivo* systems, a vascular test environment to study angiogenesis. The embryos were staged using the Hamburger and Hamilton staging for chick embryos [24]. Effect of the known angiogenic drug, VEGF, the anti-angiogenic drug, thalidomide, and Efavirenz (anti-retroviral drug) were studied.

Drug administration

Reagents were purchased sterile and equipment used were autoclaved. Treatments for all groups were given at the same time, on day 3 of incubation as follows: The blunt side of the eggs were sterilized using cotton wool soaked in 70 % ethanol and the respective drugs were administered into the air cells through a single injection, using needles and syringes. The control groups were as follows: VEGF-treated group served as the Positive control group. These subjects were treated with human recombinant VEGF protein (R&D Systems). A stock solution of 1 mg in 2 ml diluent was first prepared and a single dose of 200 µg /embryo was given [25]. Thalidomide was used as the negative control drug. A stock solution of 100 mg/ml was prepared and a single dose of 20 mg/embryo was given [26 - 27] Control 1 embryos were treated with equal volume of the diluent (1 ml of 70 % ethanol). Control 2 Embryos were not manipulated in any way. The test subjects were treated with efavirenz, obtained from Sequoia Research Products (Pangbourne, UK). A stock solution of 3 mg/ml was first prepared and treatment /embryo was 200 µg [10]. Gomez-Sucerquia ascertained that this drug has a purity of 98 – 100 % and the solutions are stable when tested by high performance liquid chromatography (HPLC). With the exception of Control 2 subjects, an observation window of 1 x 2 cm dimension was cut into the egg shells on day 5 of treatment. This was done using a sterilized flexible metal saw.
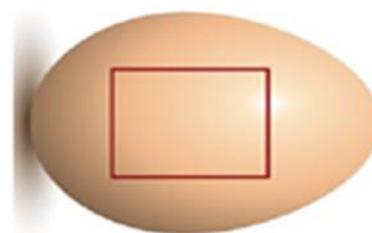

Figure 1: An illustration of the observation window cut in the egg shells

CAM sterility was maintained by instillation of 3 drops of an antibiotic agent (50 µl Penstrept in 50 ml chick ringer) into each egg window. Besides ensuring sterility, this caused the CAM to sink below the egg shell surface, for ease of embryonic CAM observation and photography. CAM images were captured using a Nikon microscope camera. The windows were then sealed with sterilized transparent tapes and the eggs were re-incubated, until day 15 of treatment when CAM images were again taken. Test results were scored based on the presence or absence of blood vessels in embryonic CAMs.

Statistical analysis

All data were analyzed using Statistical Package for Social Sciences (version 21, SPSS Inc, Chicago, IL) software program. Fisher exact test was used to determine the association between treatment drugs and growth of CAM blood vessels. One-way analysis of variance (ANOVA) was used to compare the anti-angiogenic efficacy of the drugs. $P < 0.05$ was considered significant for all analysis.

RESULTS

Mean angiogenic score

In Table 1, the results were expressed as mean angiogenic effect (n = 6). All control embryos had well-formed CAM blood vessels but the un-manipulated control subjects (control 2), shown

in Figure 2B and Figure 2D, had a 100 % angiogenicity (mean angiogenic score of 1). This was the highest embryonic viability in the experiment. VEGF treatment group (Figure 3) had the next high mean angiogenic score of 0.83. The manipulated control embryos (Control 1) in Figure 2A and Figure 2C had a score of 0.67

Table 1: Fisher's exact test results

Treatment drug	CAM blood vessel present		CAM blood vessel absent	
	f	%	f	%
VEGF	5	(31.3)	1	(7.1)
Thalidomide	1	(6.3)	5	(35.7)
Efavirenz	0	(0.0)	6	(42.9)
Control 1	4	(25.0)	2	(14.3)
Control 2	6	(35.5)	0	(0.0)

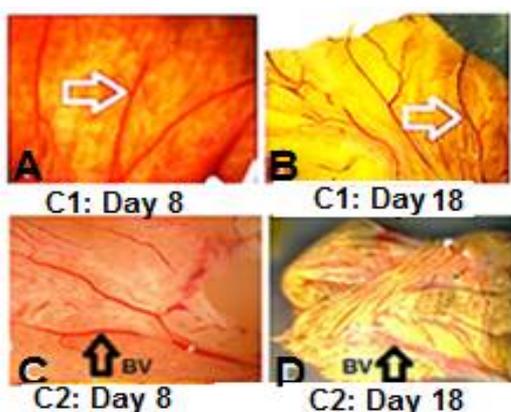*P = 0.001 (Fisher's exact test)*

Figure 2: Control embryonic CAM. Normal angiogenesis in control embryonic CAMs. C - Control. Control 1 (Figure 2A) and Control 2 (Figure 2B) showed control embryonic CAMs on day 8 of treatment. Arrows point to blood vessels on CAM surface
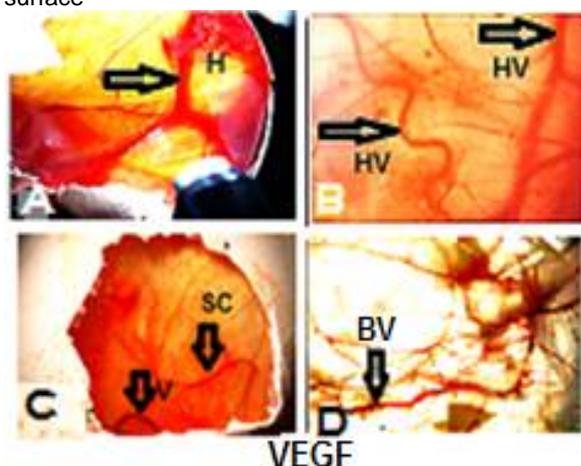

Figure 3: VEGF- treated CAMs. While images A) and B) were taken on day 8 of incubation (day 5 of treatment); images C) and D) were taken on day 18 of incubation (day 15 of treatment). In Figure 3A, arrow shows vascular haemorrhage by Day 8 of treatment

There was a significant relationship between drugs used and the growth of CAM blood vessels. Efavirenz treatment group had 42.9 % anti-angiogenicity and 0 % angiogenic effect (n = 30). This was statistically significant (p = 0.001, Fisher's exact test).

Efavirenz suppressed angiogenesis in all 6 subjects (showing absolute anti-angiogenicity and 0% angiogenicity). Thalidomide had mean angiogenicity of 0.17 and so, failed as a negative control drug in that embryo.

There was no significant difference between the anti-angiogenic effect of efavirenz and thalidomide. Although efavirenz suppressed both blood vessel development (angiogenesis) and red blood cell formation (erythropoiesis), our work did not measure the anti-erythropoietic effect of treatment drugs.

Morphological features of the embryos

Figure 2C and Figure 2D show control 1 and control 2 CAMs, respectively, on day 18 of treatment. Both control subjects had established CAM angiogenesis.

Vascular permeability is characteristic of VEGF, which is also known as a permeability factor [15]. In Figure 3B the arrow, HV showed hyper vascularization in VEGF-treated CAM on day 8 of treatment that persisted up to day 15 of treatment (Figure 3C). Note the "small caliber" blood vessels (arrow labelled SC shows). Figure 3D show CAM of an embryo that had died for an unknown reason; its CAM however had well-formed blood (see arrow labelled BV). This incidence seemed peculiar to the embryo.

When compared to control embryos, there were no established vascular network in this treatment group. But patches of erythropoietic activities were found on CAM surfaces on day 8 of treatment, as seen in Figure 4A (labeled E) and figure 4B (labeled P). By day 15, figure 4C no longer showed angiogenic activity in the CAM but instead, had excessive fibrotic tissue reaction. In Figure 4D, the embryonic CAM had scanty blood islands at the margin of the embryonic CAM (see arrows, labeled M).

Efavirenz suppressed both angiogenesis and erythropoiesis in all the animals. A rather "quiet" CAM was therefore descriptive of this treatment group. The CAM in Figure 5C had exaggerated tissue reaction. In Figure 5D, a thin fibrous structure was seen on the CAM surface.
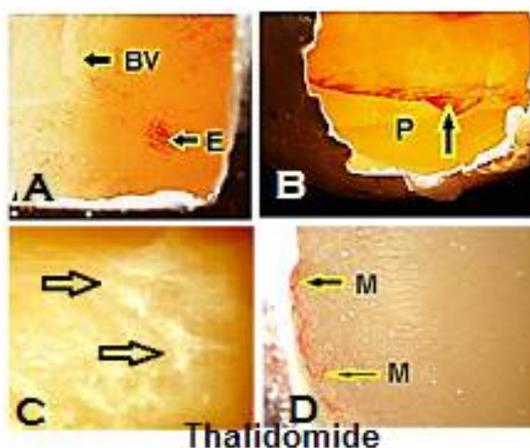
Figure 4: Thalidomide -treated CAMs. While images A) and B) were taken on day 8 of incubation (day 5 of treatment), images C) and D) were taken on day 18 of incubation (day 15 of treatment)

Figure 5: Efavirenz-treated CAMs. While images A) and B) were taken on day 8 of incubation (day 5 of treatment), images C) and D) were taken on day 18 of incubation (day 15 of treatment). No angiogenesis or erythropoiesis seen in any of the animals in this group
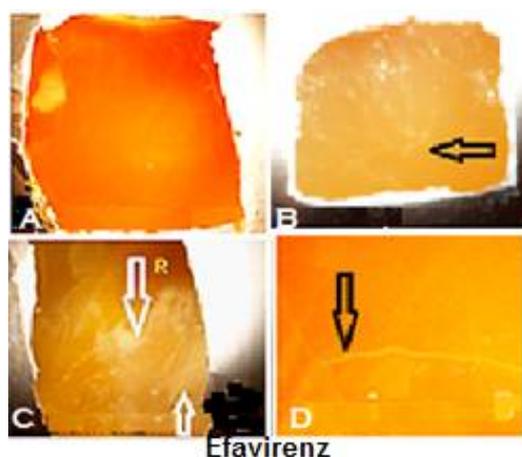
DISCUSSION

Angiogenesis begins with blood vessels forming *de novo,* from mesenchymal precursor cells called angioblasts. Angioblasts differentiate into endothelial cells that line primordial blood vessels called "blood islands". Blood islands contain progenitor blood cells called haematoblasts [2]. Both blood vessels and blood cells form from embryonic mesoderm. The blood vessels subsequently become organized into a primordial vascular network in the embryo [9].

From the results, the mean angiogenic scores showed that 5 out of 6 VEGF- treated embryos had well established CAM vessels. While vascular growth in the presence of exogenous VEGF surpassed that of Control 1(chick development with embryonic manipulation), it fell short when compared to normal uninterrupted embryonic development (as in Control 2). This suggests that exogenous VEGF did not potentiate the effect of endogenous VEGF in chick CAM. However, treatment with VEGF caused vascular permeability and haemorrhage. This agrees with the fact that VEGF is also known as permeability factor [27]. Vascular exudation causing secondary ascites has occurred in abdominal cancers because cancer cells secreted vascular permeability factor [29]. Furthermore, VEGF treatment caused hyper vascularization in some subjects (Figure 3B and 3C. This agrees with the work of Drake *et al* that high levels of exogenous VEGF caused vascular defects in a way similar to that seen with 50 % reduction in VEGF expression such as in heterozygous deletion of the VEGF allele [28]. Disorder of VEGF expression therefore causes vascular disorganization [4,21]. That is why, although VEGF use has been recommended in the treatment of ischemia and to reduce neurological deficits from stroke, it was found that an initial suppression of VEGF activity is actually necessary to combat vascular permeability and the risk of VEGF-induced hemorrhage [30].

The anti-angiogenic effect of thalidomide treatment failed in one embryo (n=6). Studies of human and animal tumours reveal that occurrence of angiogenesis is crucial in tumorigenesis. Angiogenesis is regulated by both activating and inhibitory growth factors [4]. Growth factors such as VEGF, Tumour necrotic factor (TNF), Epidermal growth factor (EGF), Transforming growth factor (TGF) and Angiogenin play key roles in tumorigenesis [4,10]. Anti-angiogenic drugs such as thalidomide suppress cancer growth and enhance patient recovery. It is now known that the resultant amelia in neonates who mothers used thalidomide during pregnancy was due to inhibition of blood vessel growth in the developing fetal limb bud [17]. It has been documented that thalidomide antagonizes angiogenic factors and is thus effective for the treatment of human cancers. From our work we found that although thalidomide -treated CAMs had poor blood vessel network, blood islands with red blood cells were seen at the periphery of CAM surfaces. Unlike thalidomide, the test drug, efavirenz, suppressed both angiogenesis and erythropoiesis in all 6 subjects. This is a significant finding that can be further explored. The 100 % anti-angiogenicity of efavirenz presents it as a potential anti-cancer drug. Distinct CAM pallor therefore characterized this drug treatment group; efavirenz had absolute anti-angiogenic effect and so, 0 % angiogenicity. It contributed 42.9 % anti-angiogenicity to the

experiment (n= 30, F = 9.306, *p* = 0.0005). Its anti-angiogenic property surpassed that of thalidomide (35.7 %). However, when we compared the anti-angiogenicity of Efavirenz to thalidomide (Table 2), there was no significant difference between the anti-angiogenicity of Efavirenz and Thalidomide (F = 1.000, *p* = 0.341, Table 3). This is because our work did not measure the anti-erythropoietic properties of the drugs.

Table 2: Mean angiogenic score

Group	Mean Angiogenic effect	Standard Deviation
VEGF	0.83	0.41
Thalidomide	0.17	0.41
Efavirenz	0	0
Control 1	0.67	0.52
Control 2	1	0

F = 9.306; p = 0.005

Table 3: Anti-angiogenic effects of efavirenz and thalidomide

Drug	N	Mean	Standard deviation
Thalidomide	6	.17	.41
Efavirenz	6	.00	.00
Total	*12*	*.08*	*.29*

F = 1.000; p = 0.341

We terminated the experiment on day 15 of treatment (day 18 of incubation). At this time, as expected, control CAM blood vessels were still well-formed. One VEGF-treated embryo died for an unknown reason since its CAM blood vessels appeared to be well-formed, 5 subjects in the group were still viable, out of which, 3 embryos had an unusually strong arterial pulsation from hypervascularization.

While one embryo in the thalidomide showed proof of life, 5 in the group and all efavirenz-treated subjects had died from lack of oxygenation and nourishment by the end of the experiment. Efavirenz CAM had severe pallor with embryonic resolution. Furthermore, there was CAM tissue thickening. Interruption of angiogenesis disrupts development of epithelial structures [11] and suppresses growth of solid tumours. Studies on inhibitors of the VEGF signaling pathway are currently being done to innovate on cancer treatment [17]. From our findings, there seems to be a promise in the use of efavirenz, beyond that of thalidomide, in the treatment of life-changing conditions such as solid tumors, diabetic retinopathy, and macular degeneration. Although both efavirenz and thalidomide - treated embryos showed signs of inadequate blood supply and nourishment,

efavirenz having shown significant anti-angiogenic and also anti-erythropoietic properties in chick CAM, has a promising future that that can be exploited in clinical practice.

CONCLUSION

The findings of this study on efavirenz indicate that it has significant anti-angiogenic effect. Being a first-line drug used in both HAART and combination treatment for HIV infection, the anti-erythropoietic and anti-angiogenic properties of Efavirenz may be responsible for spontaneous resolution of HIV-associated cancers seen in patients on HAART regimen.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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