Inhibition of Vascular Endothelial Growth Factor Synthesis in Human Retinal Pigment Epithelial Cells by Pazopanib

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Abstract: Age-related macular degeneration (AMD) is a leading cause of central vision blindness in most developed countries. Several new drugs are now available to attempt to prevent progression or treat AMD, however, they have limited effectiveness and therefore are of limited use. Human retinal pigment epithelial (hRPE) cells have been implicated in pathogenesis of AMD *via* the synthesis of vascular endothelial growth factor (VEGF), which can result in pathological proliferation of vascular endothelial cells and hRPE cells *via* tyrosine-kinase signaling pathways. Therefore, tyrosine-kinase inhibitors should be considered as potential pharmaco-therapeutic agents in the prevention and treatment of AMD. Since pazopanib (PZB) is a tyrosine-kinase inhibitor of VEGF-R1/VEGF-R2, we investigated its effect on hRPE cell proliferation and VEGF synthesis. We showed that PZB inhibited hRPE cell proliferation and VEGF synthesis in hRPE cells, and therefore it may be of therapeutic value in AMD.

Keywords: VEGF, pazopanib, hRPE, retina, AMD.

1. INTRODUCTION

Age-related macular degeneration (AMD) and associated proliferative vitreoretinopathy (PVR) affect over 2 million people in the United States, and is a leading cause of central vision loss in people over the age of 50 in developed nations worldwide [1, 2].

The human retinal pigment epithelium (hRPE) is a monolayer of epithelial cells between neural retina and choroid. hRPE cells are normally mitotically inactive after early fetal life. Referred to as “the liver of the eye”, hRPE cells play many roles in supporting the normal functioning of the photoreceptor cells. They are integral to the metabolism and transport of nutrients to and from the neural retina, disposal of waste, and protection of the photoreceptor cells from oxidative stress.

AMD is associated with the development of choroidal neovascularization and abnormal vitreoretinal proliferation. Since hRPE cells are also a major source of many angiogenic factors, such as vascular endothelial growth factor (VEGF), they may play a role in the development of these complications, and inhibiting overproduction of VEGF by hRPE may have therapeutic value in prevention or treatment [3].

Since pazopanib (PZB) is a tyrosine-kinase inhibitor of VEGF-R1/VEGF-R2 and has been proven to be an effective treatment for various types of cancer, we investigated if it has any effect on hRPE cell proliferation and VEGF synthesis [4].

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2. MATERIALS AND METHODS

2.1. Cell Proliferation Measured by the Trypan Blue Exclusion Method

Normal human eyes were obtained from the Michigan Eye Bank. hRPE cells were isolated as described by Weng *et al.* and Kothary *et al.* [5, 6]. The cells were then plated in 6-well plates in Ham's F-12 nutrient media and incubated at 37ºC in a 5% CO_2 incubator. After establishing primary cultures near confluence, supernatant media in the wells was aspirated and cells were washed with 2.5 mL Ham's F-12. Then, cells were again treated with 2.5 mL F-12, and were allowed to sit at room temperature for 15 min. This medium was discarded and 2.5 mL of each of the following reagents were added to each well, respectively: Control (F-12), PZB (10.0 mM), PZB (5.0 mM), PZB (1.0 mM), PZB (0.5 mM), and PZB (0.1 mM). The cells were then incubated at 37ºC for 48 h. The cells were then washed with 1.0 mL of cold PBS and treated with 1.0 mL of Hank's Buffer (Ca^{2+} and Mg^{2+} free) followed by 750 µL of Trypsin added to each well. Subsequently, cells were incubated for 10 min at 37ºC and then viewed under a microscope to ensure detachment. Once the cells were observed to be detached, 10 µL of filtered Trypan Blue was added to each well. Viable trypan blue excluding cells were directly counted in a hemocytometer.

2.2. Immunoprecipitation

Cultured hRPE cells were plated in 12 of the wells in 24-well plates, incubated at 37ºC, and allowed to attach to the plate. The supernatant media in the wells

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was aspirated and cells were washed with 0.5 mL Ham's F-12. Cells were then treated again with 0.5 mL F-12, and allowed to sit at room temperature for 15 min, after which media were replaced with 0.5 mL of media containing each of the following reagents added to each experimental well: Control (F-12), PZB (10.0 mM), PZB (5.0 mM), PZB (1.0 mM), PZB (0.5 mM), and PZB (0.1 mM). Cells were then incubated for 1 h at 37ºC. Subsequently, 100 µL of 14C-Methionine was added to each well. Cells were again incubated at 37ºC for 24-48 h after which the media were aspirated and the cells were washed with 0.5 mL PBS. The PBS was then replaced with 200 µL of Zwitteragent in BSA. Next, 10 µL of Anti-VEGF were added to each well, and the entire plate was placed in the refrigerator at 4ºC for 24-48 h. After the refrigeration, 10 µL of Protein A were added to each well, whereupon cells stayed in room temperature for an hour, after which the cells were centrifuged and the supernatant media were aspirated. Finally, 0.5 mL of NaOH and 10 mL of Ecolite was added to the cells. After one hour, a cell count was obtained using a scintillation counter.

2.3. Immunocytochemistry

hRPE cells were plated in 3 of the wells in a 6-well plate and incubated at 37ºC. The supernatant media was aspirated and then the cells were washed with 2.5 mL Ham's F-12. After this was aspirated, cells were again treated with F-12, and allowed to sit at room temperature for 15 min. Once this was discarded, 2.5 mL of each of the following reagents were added to each well, respectively: Control (F-12), PZB (10.0 mM), PZB (10.0 mM) + VEGF-R2. Cells were then incubated at 37ºC for 24-48 h. Next, the media was aspirated and 2 mL of PBS was used to wash the cells (twice). Then, 2 mL of 4% formaldehyde in PBS was added to each well. After 20 min, the formaldehyde was aspirated and cells were washed with PBS again (twice). Subsequently, 1-2 mL of Zwitteragent in BSA was added to each well. After an hour, the Zwitteragent in BSA was aspirated and 3mL of PBS was added. Then, 1.5 mL of anti-VEGF-R2 was added. Cells were then placed in the refrigerator for 24 h. After the refrigeration at 4ºC, the antibody was aspirated and wells were washed twice with 1-2 mL Zwitteragent in BSA. Then, 1-2 mL of PBS was used to wash and cleanse the cells. Next, 1.5 mL anti-rabbit IGG-Rhodamin (30 µL anti-rabbit IGG-Rhodamin + 3 mL Zwitteragent in BSA) was added to the cells and allowed mix at room temperature. After one hour, the anti-Rhodamin was aspirated and cells were washed twice with 1-2 mL of Zwitteragent in BSA. They were then washed twice with 1-2 mL of PBS, after which microscope slides were used to mount the cover slips containing the cells by using a drop of gel/mount, 4',6-diamidino-2-phenylindole (DAPI). Finally, the slides with the cells were placed in 4ºC, and later visualized and photographed under a Nikon Eclipse e800 microscope.

2.4. Materials

FBS and F-12 were purchased from Fischer Scientific in Ann Arbor, MI. Pazopanib was obtained from LC laboratories in Woburn, MA. VEGF was acquired from RD Systems in Minneapolis, MN. 14C methionine and 3H Thymidine were obtained from Amersham Corporation in Arlington Heights, IL.

2.5. Statistical Analysis

Differences between the two groups of data were tested by student's t-test. A p-value of <0.05 was used to assess statistical significance.

3. RESULTS

Figures **1** and **2** show that increasing concentrations of fetal bovine serum (FBS) stimulate hRPE cell proliferation and maintain cell viability as shown by T and 3H-Thy.

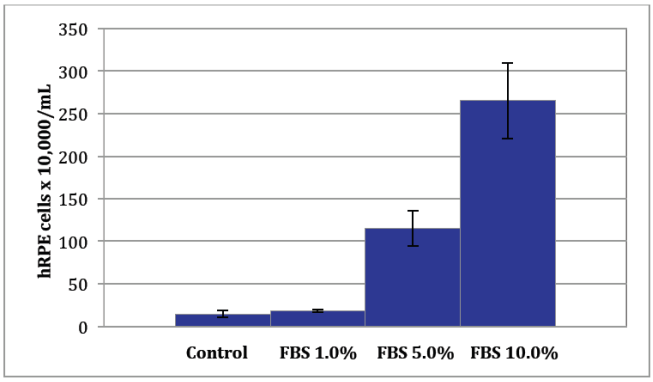


Figure 1: Trypan blue exclusion method, effect of fetal bovine serum on hRPE cell viability.

Figure **3** shows that PZB inhibits hRPE cell proliferation in a dose dependent manner. PZB (10 mM) inhibits T (4.88±2.30 vs. 10.75± 2.20, cells per 0.1 µl ±SEM, $p \leq 0.05$, n=8).

Figure **4** shows that PZB (0-10 mM) also inhibits immunoprecipitated 14C-VEGF synthesis in a dose dependent manner.

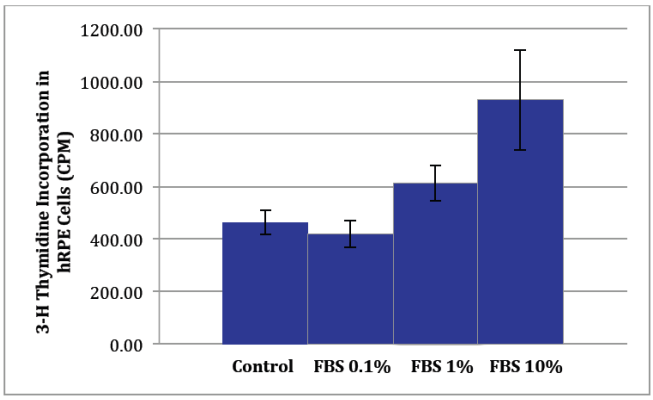


Figure 2: 3H-Thymidine incorporation, effect of fetal bovine serum on hRPE cell viability.

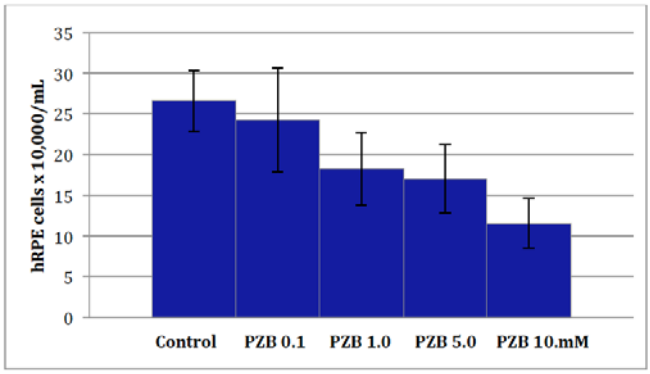


Figure 3: Trypan blue exclusion method, effect of pazopanib on hRPE cell viability.

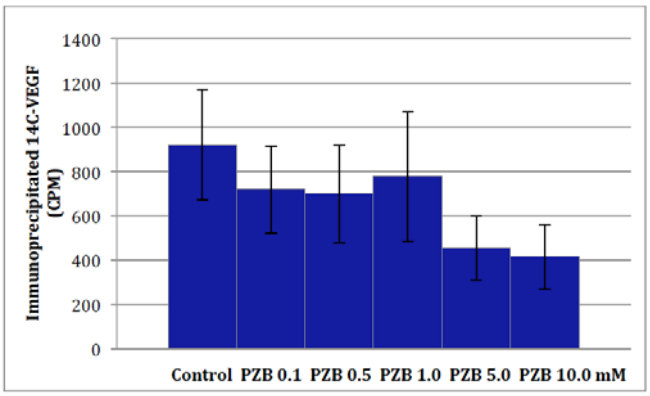


Figure 4: Immunoprecipitation, effect of pazopanib on 14C-VEGF synthesis in hRPE cells.

Qualitative studies using nuclear staining of hRPE cells by DAPI demonstrate that PZB altered hRPE morphology resulting in smaller dysplastic cells (Figure **5**). Immunocytochemical analysis confirms that PZB inhibits VEGF synthesis in hRPE cells (Figure **6**).

4. DISCUSSION

Age related macular degeneration is a progressive disorder of the retina, and remains one of the leading causes of blindness in the elderly worldwide. It is classified into geographic atrophy (dry AMD) or exudative (wet AMD) [1]. Although wet AMD affects only 10-15 percent of those who have the condition, it accounts for 90 percent of the severe vision loss caused by macular degeneration. Moreover, while there are no medically proven treatments for dry AMD, there are several treatment options for wet AMD related to its pathogenesis [2].

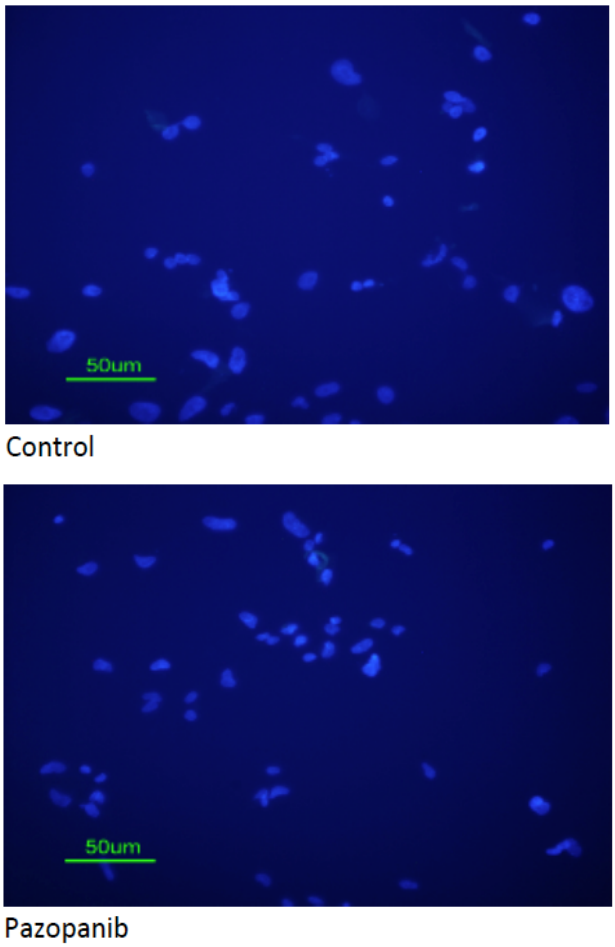
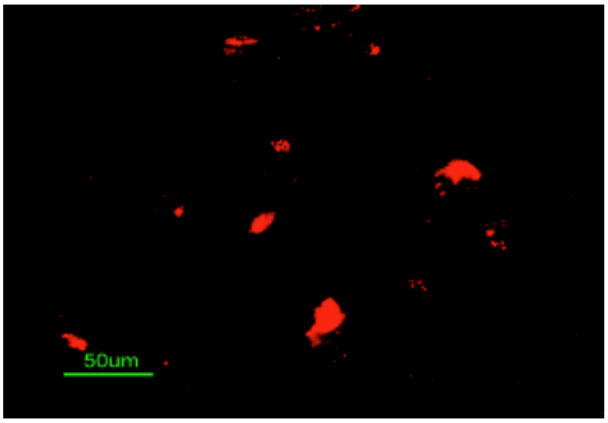


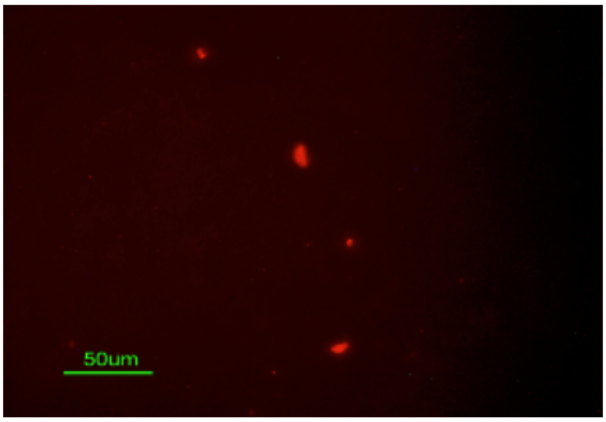
Figure 5: Nuclear staining (DAPI) of hRPE cells in presence/absence of pazopanib (10 mM).

Wet AMD is characterized by choroidal neovascularization—the formation of abnormal blood vessels—which leads to accumulation of sub retinal fluid in the macular area, retinal or vitreous hemorrhage, and possible macular detachment and vision loss. This process of choroidal neovascularization is associated with increased levels of VEGF (vascular endothelial growth factor), a known ocular mediator of angiogenesis [7,8,9,10,15].

The pathway of VEGF is receptor tyrosine kinase mediated, and involves a Ras/Raf signaling pathway.



Control



Pazopanib

Figure 6: Immunocytochemistry, effect of pazopanib (10mM) on VEGF expression.

There are 3 receptor tyrosine kinases, VEGFR1, VEGFR2, and VEGFR3, each with their respective ligands, which include VEGF-A, VEGF-B, VEGF-C, and VEGF-D, VEGF-E, and PIGF. Although there are many combinations of ligands and receptors, the angiogenic effects of this pathway are predominantly due to VEGF-A (often simply called VEGF), and VEGFR2 [3,12].

hRPE cells are a known source of angiogenic factors such as VEGF, and their pathological metaplasia and pathological proliferation in later life are linked to several retinal diseases such as AMD [12]. In a healthy eye, the hRPE cell layer provides nutrients to the overlying retina, and also acts as a blood-retina barrier. However, their pathologic proliferation can lead to fibrosis, membrane formation/scarring, hemorrhage and vascular leakage, and eventual vision loss.

Current FDA approved therapies for wet AMD include anti-VEGF therapies administered *via* intra-vitreal injections. Pegaptanib is a selective $VEGF_{165}$ inhibitor (a prominent variant of VEGF-A), and is primarily used to prevent further vision loss. Ranibizumab is a recombinant humanized monoclonal antibody fragment derived from mouse monoclonal antibody—it binds and inhibits VEGF-A, and is used to improve vision [13,14].

Pazopanib is a tyrosine kinase inhibitor which is currently FDA approved for the treatment of renal carcinoma [4]. It works by inhibiting angiogenesis, and acts on VEGFR1 and VEGFR2.

Our study demonstrates that pazopanib inhibits VEGF synthesis within hRPE cells. As a known VEGF inhibitor, these results were not surprising. However, pazopanib also inhibits hRPE cell proliferation, which is associated with AMD pathogenesis. These findings suggest that pazopanib may be a useful pharmaco therapeutic agent for the prevention or treatment of the blinding vasoproliferative and fibrotic complications in AMD.

In conclusion, we show that pazopanib inhibits VEGF synthesis within hRPE cells, and inhibits hRPE cell proliferation. Since wet AMD is linked to hRPE cell proliferation in later life, and the subsequent expression of VEGF, pazopanib may be a useful therapeutic agent worthy of further study to prevent progression or treat this blinding condition.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

ACKNOWLEDGEMENT

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