Verteporfin without light stimulation inhibits YAP activation in trabecular meshwork cells: Implications for glaucoma treatment

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ABSTRACT

Verteporfin, a photosensitizer, is used in photodynamic therapy to treat age-related macular degeneration. In a glaucoma mouse model, Verteporfin without light stimulation has been shown to reduce intraocular pressure (IOP) but the mechanism is unknown. Recent studies have shown that Verteporfin inhibits YAP without light stimulation in cancer cells. Additionally, YAP has emerged as an important molecule in the pathogenesis of glaucoma. We hypothesize that YAP inactivation by Verteporfin in trabecular meshwork (TM) may be related to the reduced IOP observed in vivo. As contractility of TM tissues is associated with IOP, collagen gel contraction assay was used to assess the effect of Verteporfin on contractility of TM cells. Human TM cells were embedded in collagen gel and treated with Verteporfin for 48 h. Areas of collagen gel sizes were quantified by ImageJ. To assess the effect of Verteporfin on the expression of YAP, human TM cells were treated with Verteporfin for 24 h and the expression of YAP was determined by Western blotting. To determine the cytotoxic effect of Verteporfin, human TM cells were treated with Verteporfin for 24 h, and then the cell viability was assessed by WST-1. We demonstrated here that Verteporfin (i) abolishes TM cell-mediated collagen gel contraction in a dose-dependent manner, (ii) attenuates expression of YAP and CTGF (connective tissue growth factor, a direct YAP target gene) in a dose-dependent manner, and (iii) has no significant cytotoxicity below 2 μM. Taken together, Verteporfin may facilitate aqueous humor outflow through the conventional outflow system and reduce IOP by inactivating YAP.

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1. Introduction

Glaucoma is the second leading cause of blindness, affecting over 60 million adults worldwide [1,2]. More than 3 million Americans age 40 and older, about 2% of this population, have glaucoma. Glaucoma is estimated to account for $US2.9 billion of direct medical costs among US adults annually [3]. Currently, treatment for glaucoma is designed to lower intraocular pressure (IOP), as this is the only modifiable factor that has been shown to slow the progression of glaucoma. IOP is determined by the balance between aqueous production and aqueous outflow. Aqueous drainage through the trabecular meshwork (located at the junction of the cornea and the iris) and Schlemm's canal (i.e. the conventional outflow pathway) accounts for a large portion of aqueous outflow in human [4,5]. There are few medications targeting the conventional pathway; the majority of the current glaucoma medications target aqueous production and/or the non-conventional (uveoscleral) outflow pathway. Significant efforts have been made to enhance outflow through the conventional pathway. Several therapeutic agents targeting this pathway are currently under investigation [6,7].

Verteporfin is used as a photosensitizer for photodynamic therapy and was approved by the Food and Drug Administration to treat neovascularization caused by age-related macular degeneration, pathologic myopia, and presumed ocular histoplasmosis. In a glaucoma mouse model [8], Verteporfin with light stimulation targeting ciliary body markedly reduced IOP. Interestingly, the IOP of mice treated with Verteporfin alone was also significantly reduced. While the authors noted this finding, the mechanism, to our knowledge, was not proposed. A recent high-throughput screening showed that Verteporfin inhibits YAP activation

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without light stimulation [9]. YAP is a mechanotransducer that "reads" physical and mechanical cues from cellular surroundings. It has been demonstrated that elevated stiffness of extracellular matrix induces the interaction of nuclear YAP and TEAD (a transcriptional factor) in cancer-associated fibroblasts [10]. Studies have also shown that YAP–TEAD interactions upregulate several genes that are associated with glaucoma, including connective tissue growth factor (CTGF), transglutaminase-2, plasminogen-activator inhibitor-1, and transforming growth factor-β (TGF-β) [11,12], all of which play important roles in modulating the homeostasis of the extracellular matrix of the TM. Considering that glaucomatous TM is stiffer than normal TM [13] and that YAP–TEAD interactions regulate the gene expression of several extracellular matrix-modifying factors, targeting YAP or the YAP–TEAD interaction appears to be an attractive strategy to regulate the functions of TM.

We demonstrate here that Verteporfin inhibits the expression of YAP with a concomitant inhibition of the contractility of TM cells in a dose-dependent manner. Given that contraction and relaxation of TM tissue are thought to control IOP [14–20], our results suggest that Verteporfin without light stimulation holds promise in altering TM function. This may, in turn, reduce IOP by targeting YAP and facilitating aqueous humor outflow through the conventional outflow pathway.

2. Materials and methods

2.1. Human TM cell cultures

Primary human TM cells were purchased from ScienCell Research Laboratories. These cells can form typical cross-linked actin networks after dexamethasone treatment [21]. In addition, primary human TM cells (clone NTM88) isolated from human donors as described in our previous studies [22–24] were used in this study. Cultures were propagated in DMEM supplemented with 10% FBS. Same results were obtained by using the two different sources of TM cells.

2.2. Cytotoxicity assay

To assess the cytotoxicity of Verteporfin in 2D monolayer cultures, human TM cells (ScienCell Research Laboratories, Carlsbad, CA) (10,000 cells in 100 μl of 10% FBS/DMEM) were seeded in a 96-well plate and cultured at 37 °C overnight. The cells were treated with different concentrations of Verteporfin (0–5 μM) in 0.2% DMSO/1% FBS/DMEM and cultured at 37 °C overnight. Ten μl of WST-1 reagent (Cayman Chemical, Ann Arbor, MI) was added to each well of the 96-well plate and incubated at 37 °C for 2 h. The absorbance of each sample was measured using the FilterMax F5 microplate reader (Molecular Devices, Sunnyvale, CA) at a wavelength of 450 nm.

To assess the cytotoxicity of Verteporfin in 3D spheroid cultures, human TM cells (5000 cells in 50 μl of 10% FBS/DMEM) were added to each well of a 96-well hanging drop plate (3D Biomatrix, Ann Arbor, MI) and cultured at 37 °C overnight to form spheroids. Five μl of 10X treatments (vehicle and Verteporfin in serum-free FBS/DMEM) was added to each well of the 96-well hanging drop plate and cultured at 37 °C overnight. The spheroids were collected by placing the 3D culture plate on top of a 96-well round bottom clear plate and centrifuging at 200× g for 1 min. Five μl of WST-1 reagent was added to each well of the 96-well round bottom clear plate and incubated at 37 °C for 3.5 h. The absorbance of each sample was measured using the FilterMax F5 microplate reader (Molecular Devices) at a wavelength of 450 nm.

2.3. Collagen gel contraction assay

To assess the effect of Verteporfin on TM cell-mediated gel contraction, the collagen gel contraction assay was performed as previously described [25]. Briefly, type I collagen (PureCol, 3 mg/ml, Advanced BioMatrix, San Diego, CA), 10 × DMEM (Invitrogen, Grand Island, NY), reconstitution buffer (262 mM of NaHCO₃, 200 mM of HEPES and 0.05 N of NaOH), TM cell suspension (2.5×10^5 cells/ml) and deionized water were mixed at a ratio of 7:1:1:1:1. The resultant mixture (0.5 ml) was added to each well of a 24-well plate in the presence or absence of Verteporfin. After incubation at 37 °C for 1 h, vehicle (control) or Verteporfin in 0.5 ml of serum-free DMEM was added to each well, and the gels were freed from the walls of the culture wells with a microspatula. After 2 days, images of the collagen gels were acquired with the SynGene imaging system (Frederick, MD) using GeneSnap software (Frederick, MD). The areas of collagen gels were measured by ImageJ. The extent of TM cell-mediated gel contraction was calculated by the formula: (area of the well − area of the gel)/area of the well, where the value of control group was set as 100%.

2.4. Western blot analysis

Protein extracts of human TM cells were prepared in a radioimmunoprecipitation (RIPA) buffer supplemented with a protease inhibitor cocktail (cOmplete tablets, Roche Applied Science, Indianapolis, IN) and a phosphatase inhibitor cocktail (PhosSTOP, Roche Applied Science). Aliquots of cell lysates containing 25 μg of proteins were subjected to electrophoresis in 4–15% SDS-PAGE gels (Bio-Rad, Hercules, CA). Protein blots of the gels were blocked with Odyssey's blocking buffer (OBB, 30 min, 25 °C, LI-COR, Lincoln, NE), incubated with primary antibodies (rabbit anti-phospho-YAP, Ser127, 1:1000 dilution, Cell Signaling, Danvers, MA; mouse anti-YAP, clone 63.7, 1:1000 dilution, Santa Cruz Biotech, Dallas, TX; goat anti-CTGF, clone L-20, 1:500 dilution, Santa Cruz Biotech; mouse anti-GAPDH, clone 6C5, 1:10,000 dilution, Santa Cruz Biotech) in OBB (overnight, 4 °C) and secondary antibodies (donkey anti-rabbit IgG 800CW, anti-goat IgG 800CW, and anti-mouse IgG 680LT, 1:10,000 dilution) in OBB (45 min, 25 °C). Blots were then scanned with the Odyssey Infrared Imaging System using Image Studio v2.0 software (Li-COR). Relative band intensity was quantified by Image Studio v2.0 software.

2.5. Statistics

Data were analyzed using an unpaired 2-tailed Student t test in Prism 6 (GraphPad). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Cytotoxicity effect of Verteporfin in human TM cells

We first examined whether Verteporfin without light stimulation is cytotoxic to human TM cells. Viability of human TM cells was assessed by WST-1 reagent, a tetrazolium salt which is converted to soluble formazan by mitochondrial and plasma membrane dehydrogenases. In 2D monolayer cultures, Verteporfin at 2 μM or below had no significant effect, whereas Verteporfin at 5 μM markedly reduced viability of human TM cells (Fig. 1A). As cells in 3D tissue-like cultures have different response to drugs compared to 2D monolayer cultures [26], we also assessed viability of human TM cells in 3D spheroid cultures in response to varying doses of Verteporfin. Interestingly, viability of human TM cells in 3D spheroid cultures was increased at concentrations greater than 1 μM of Verteporfin (Fig. 1B).