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Effects of white light on β-catenin signaling pathway in retinal pigment epithelium

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ABSTRACT

This study investigated the effect of visible light exposure on retinal pigment epithelium (RPE). The activation of Wnt/β-catenin pathway was investigated by immunofluorescence and Western blot analysis using human retinal pigment epithelial (ARPE-19) cells, which demonstrated that the exposure of white light induced the activation of the Wnt/β-catenin pathway. Real time RT-PCR demonstrated that the mRNA of α-smooth muscle actin (α-SMA), and vimentin increased 2.5–4-fold and that of zona occludens 1 (ZO-1) decreased approximately 0.8-fold after white light exposure. The up-regulation of vimentin expression and the down-regulation of ZO-1 were evident by Western blot analysis and immunohistochemistry. Moreover, the ability of phagocytosis of ARPE-19 cells decreased 0.6-fold after light exposure. Together, white light exposure was supposed to induce the activation of Wnt/β-catenin pathway, the changes in the expression markers of epithelial and mesenchymal cells in RPE cells, and the concomitant impairment of the ability of phagocytosis.

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Age-related macular degeneration (AMD) is the leading cause of legal blindness in developed countries [1]. The pathogenic mechanisms in which environmental factors contribute to AMD remain elusive. However, recent studies have provided evidence that oxidative stress injury to the retinal pigment epithelium (RPE) is to be implicated in the pathogenesis of AMD [2–4]. The RPE is at high risk of oxidative injury due to the high level of light exposure at its location, and the generation of reactive oxygen species (ROS) from the photoreceptor outer segment (POS) [5]. In the early stage of AMD development, oxidative stress induces a set of profound physiological responses in the RPE, leading to dysfunction without initiation of cell death [6]. Interestingly, recent studies suggest that visible light exposure induces oxidative stress to the RPE cells [7]. Although it remains unsubstantiated, several epidemiological studies have demonstrated that visible light exposure is related to the progression of AMD [4]. Laboratory studies have demonstrated that light exposure affects the viability of RPE cells [3] however, little is known about the effects of sublethal light exposure on RPE functions such as blood-retinal barrier, phagocytosis and phagolysosomal degradation.

The Wnt/β-catenin signaling pathway provides key regulations in a range of biological processes including cell motility, differentiation, and tumorigenesis [8,9]. Wnt acts in a paracrine fashion on cells expressing Frizzled receptors which in turn recruit and activate the cytoplasmic phosphoprotein Dishevelled (Dvl). Dvl transduces signals to downstream components, leading to the stabilization of β-catenin and subsequent activation of the transcription factor T cell factor (TCF) and/or lymphoid enhancer factor (LEF). It is generally accepted that the activation of β-catenin in epithelial cells leads to epithelio-mesenchymal transition (EMT). Recent studies have revealed that nucleoredoxin (NRX) interacts with Dvl in a redox sensitive manner, and redox-dependent regulation of β-catenin signaling through NRX contributes to cell proliferation induced by oxidative stress [10,11]. Several groups revealed that mice deficient in Cu, Zn-superoxide dismutase (SOD2), which is one of the main anti-oxidative enzymes in the retina, have features typical of AMD, and that in SOD1 deficient mice, their β-catenin-mediated cellular integrity was disrupted and showed some features that resembled AMD [12–14]. Notably, in SOD-1 knockout mice, in which β-catenin disruption was observed, abnormal cellular deposit formation was observed presumably due to the functional loss of RPE cells [14]. These studies support a link of β-catenin and AMD.

This study investigated whether the exposure of white light activates the β-catenin pathway, the change in the markers of epithelial and mesenchymal on RPE cells and the effects of the phagocytotic activity of RPE.

Materials and methods

Spectral analysis and power output of light source. In this study, an in-house built light source, equipped with 96-white light

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emitting diodes (LED) fixed into each well of a 96-well plate was used. Spectral irradiance was measured with a spectrophotometer, shown in Fig. 1A. Measurements were taken from 300 to 1000 nm at 10 cm from the light source. The total irradiance was 143 μW/cm^2. To confirm that the cells were evenly exposed to the light, the measurements were taken from several points and the irradiance in the darkest field was 82% of that in the brightest field.

Cell culture and white light exposure. Human ARPE-19 cells (American Type Culture Collection, Manassas, VA) were used in this study. Cells were grown in 1:1 mixture (vol/vol) of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12 medium (DMEM F-12; Invitrogen-Gibco, Carlsbad, CA), nonessential amino acids 10 mM 1×, 0.37% sodium bicarbonate, 0.058% L-glutamine, 10% fetal bovine serum, and antibiotics (penicillin G 100 U/mL, streptomycin sulfate 0.1 mg/mL, gentamicin 10 μg/mL, and amphotericin B 2.5 μg/mL). Cells were cultured at 37 °C in a humidified atmosphere with 10% CO_2. The cells were grown to confluence and 48 to 72 h after confluence; the cells were exposed to the LED, which was positioned at 10 cm above the cell, for the indicated hours. In order to exclude the possible effects of temperature increase by light exposure [15], the LED light was placed either at 1, 2.5, and 10 cm above the cells and temperature of the medium was monitored at 1, 2, 3, and 4 h after the cells were exposed from the LED light, which demonstrated that under the current condition, the temperature of the medium was kept at 37 °C throughout the measurements (Fig. 1B).

Cell proliferation assay. To examine the effects of the white light exposure on the proliferation and survival of RPE cells, WST-1 colorimetric assay (Roche Diagnostics, Indianapolis, IN) was performed, as previously described [16]. Briefly, 10 μl of the formazan dye was added to each well containing cells and medium and incubated for 1 h at 37 °C. Absorbance was measured at 405 nm on a multiwell spectrophotometer (Perkin-Elmer, Wellesley, MA).

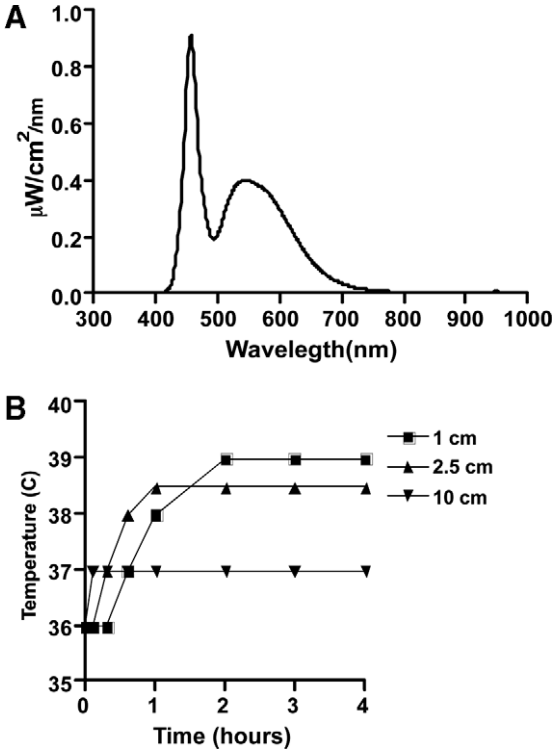

Fig. 1. Spectral analysis and power output of the light source. (A) Spectral irradiance was measured with a spectrophotometer. Measurements were taken from 300 to 1000 nm at 10 cm from light source. (B) The LED light was placed 1, 2.5, and 10 cm above the cells and temperature of the medium was taken at 1, 2, 3, and 4 h after the cells were exposed from the LED light. Note that the temperature of the medium was kept at 37 °C throughout the measurements when the light source was placed 10 cm above the cells. A representative result from two independent experiments is shown.

RPE phagocytosis. ARPE-19 cells were seeded onto 96-well plates and were grown to confluence. To investigate the phagocytotic activity in this study, pHrodo™ *Escherichia coli* BioParticles® (Molecular Probes) were used in accordance with the manufacturer's instructions. Briefly, after the cells were exposed to white light for 48 h, the culture medium was replaced with 100 μL of the prepared pHrodo™ BioParticles® suspension. The cells were then incubated for 2 h at 37 °C in a humidified atmosphere with 10% CO_2. To measure the quantity of the internalized particle, measurements of fluorescence were taken by using a multiwell spectrophotometer (Perkin-Elmer, Wellesley, MA).

Reverse transcriptase polymerase chain reaction (RT-PCR). RNA for RT-PCR was isolated using an SV Total RNA Isolation Kit (Promega, Madison, WI) in accordance with the manufacturer's instructions. cDNA was prepared using Superscript III for RT-PCR (Invitrogen). Each PCR was carried out in a 20 μl volume using Platinum SYBR Green qPCR SuperMix UDG (Invitorogen) for 15 min at 95 °C denature, followed by 45 cycles at 95 °C for 30 s and 60 °C for 1 min in Roche LightCycler. Values for each gene were normalized to expression levels of GAPDH. The sequences of the primers used for RT-PCR were as follows; human-GAPDH, left, 5′-gagtcaacggat ttggtcgt-3′, right, 5′-ttgattttggagggatctcg-3′. human-vimentin, left, 5′-gagaactttgccgttgaagc-3′, right, 5′-tccagcagcttcctgtaggt-3′. human-α-smooth muscle actin (α-SMA), left, 5′-atcaccatcggaaatga a cg-3′, right, 5′-ctggaaggtggacagagagg-3′, human-zona occludens 1 human-ZO-1 left, 5′-ccagaatctcggaaaagtgc-3′, right, 5′-accgtgtaa tggcagactcc-3′. The results were expressed as means from six independent studies.

Western blot analysis. Two hours after light exposure, cells were lysed with ice-cold RIPA buffer (50 mM Tris–HCL (PH 7.4), 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, and 1 mM EDTA). The cell lysates were centrifuged for 15 min at 14,000 rpm in a microcentrifuge. Protein content of the resultant supernatants was measured by using BCA Protein assay kit. Protein samples were dissolved in sample buffer at a concentration of 1:1. Samples were resolved by SDS–PAGE and electroblotted onto a polyvinylidene difluoride membrane. The membrane was placed in blocking solution (5% non-fat dry milk in TBST) for 1 h at room temperature. The membranes were incubated overnight at 4 °C with anti-β-catenin (Santa Cruz biotechnology) 1:200, anti-phosphorylated-β-catenin (Santa Cruz Biotechnology) 1:200, anti-vimentin (abcam) 1:2000, anti-ZO-1 (ZYMED Laboratories) 1:100. The membranes were incubated with horseradish-peroxidase labeled second antibody (Amersham Biosciences) 1:5000 for 1 h at room temperature. The membranes were developed with ECL Plus Western Blotting Detection Reagents (General Electric Company).

Immunocytochemistry. Approximately 1×10^6 cells/cm^2 were seeded onto 24-well slides, grown to confluence and were exposed to the LED for 48 h. Immunocytochemistry was performed as described [17,18]. Briefly, chamber slides were rinsed in PBS and fixed with 4% paraformaldehyde in TBS for 20 min. Then, the slides were blocked with goat serum, and stained with primary antibody to α-SMA (1:1000), vimentin (1:1000) or ZO-1 (1:100) overnight at 4 °C. The slides were rinsed and incubated Alexa488-conjugated secondary antibody for 1 h at room temperature. Nuclei were counterstained with DAPI. Stained cells were visualized with a microscope (OLYMPUS DP71, Tokyo, Japan). Negative control experiments were performed without primary antibodies, which gave no positive signals. At least three independent experiments were performed, each of which gave similar results.

Results

The exposure of white light induces the activation of Wnt/β-catenin pathway

In normal conditions, β-catenin is associated with cadherins at the plasma membrane to promote cell adhesion. The level of cytoplasmic β-catenin is normally controlled by formation of a degradation complex, which leads to phosphorylation of β-catenin and subsequently to degradation mediated by the E3 ubiquitin ligase β-TrCP [8,9]. When β-catenin pathway is activated, the formation of this degradation complex is inhibited, which leads to stabilization of β-catenin. Stabilized β-catenin translocates to the nucleus where it interacts with TCF/LEF transcription factors resulting in activation of β-catenin pathway target genes.

Western blot analysis and immunocytochemistry were performed to examine whether the exposure of white light induces the activation of Wnt/β-catenin pathway. First, Western blotting analyses were performed to examine the β-catenin phosphorylation, after white light exposure for 2 h. The results shown in Fig. 2 demonstrate that the expression of phosphorylation of β-catenin had decreased after white light exposure. Next, the localization of β-catenin was examined by immunocytochemistry. The results demonstrated the nuclear translocation of β-catenin after white light exposure using ARPE-19 cells (Fig. 2B, arrowheads). The results indicated that the white light exposure lead to the activation of β-catenin in RPE cells.

The effect of a major component of lipofuscin, A2E, a well-studied endogenous photosensitizer in RPE cells [16,19], was also investigated as to whether A2E exposure to light leads to activation of β-catenin; however, the results demonstrated that in the presence of A2E alone, the β-catenin activation level (loss of phosphorylated and intracellular translocation of β-catenin) was observed to be similar to that observed after the white light exposure and additive additional effect was not detected under the current condition (Fig. 2 and data not shown).

Loss of epithelial markers and acquisition of mesenchymal markers in ARPE-19 cells after white light exposure

β-Catenin activation affects cellular proliferation. Thus, WST-1 assay was performed to examine the effect of white light exposure on the proliferation of RPE cells. The results demonstrated that when the ARPE-19 cells were exposed to LEDs for 48 h, the cell viability was not different from the cells that were not exposed to light (data not shown).

Another important function of β-catenin activation is on cellular differentiation. β-Catenin transactivates many EMT markers. To further confirm the effect of β-catenin activation in RPE cells, the mRNA levels of the mesenchymal markers, i.e., vimentin and α-SMA and the epithelial marker ZO-1, were examined by means of

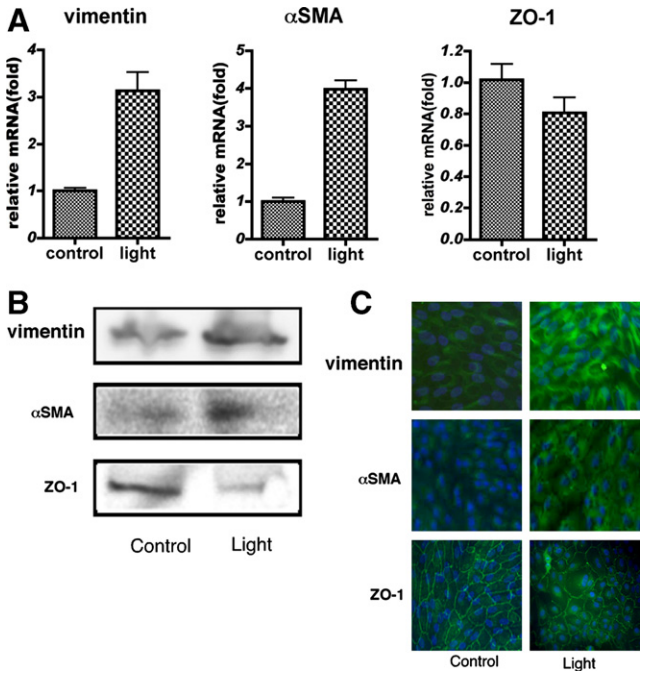

Fig. 3. Loss of epithelial markers and acquisition of mesenchymal markers in ARPE-19 cells after exposure of white light. (A) RT-PCR analyses of quantitatively standardized total RNA sample from ARPE-19 cells at 48 h after white light exposure. Quantitative PCR results for vimentin, aSMA and ZO-1 expression normalized to GAPDH. The mRNA of α-SMA and vimentin were increased 2.5–4-fold and that of ZO-1 were decreased approximately 0.8-fold. (B) Western blot analysis of whole-cell extracts prepared from ARPE-19 cells after white light exposure for 48 h demonstrate the increased expression of vimentin, α-SMA and the decreased expression of ZO-1. (C) Immunocytochemistry of ARPE-19 cells after white light exposure for 48 h. Cell were fixed with paraformaldehyde and stained with antibody against for vimentin, α-SMA and ZO-1. Nuclei were counterstained with DAPI.

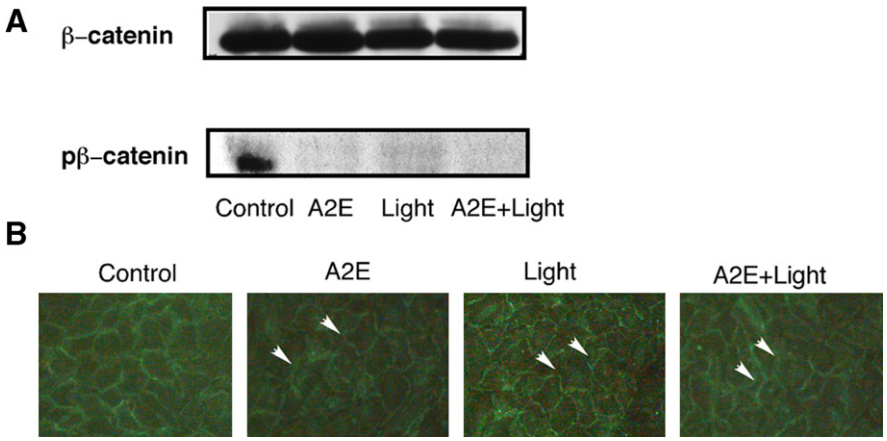

Fig. 2. The exposure of white light induced the activation of Wnt/β-catenin pathway. (A) Western blot analysis of whole-cell extracts prepared from ARPE-19 cells after white light exposure for 2 h demonstrates that the expression of phosphorylation of β-catenin was decreased. (B) Immunocytochemistry of ARPE-19 cells after white light exposure for 2 h demonstrate that translocation of β-catenin (arrowheads).

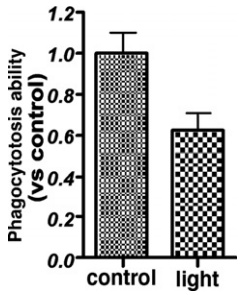

Fig. 4. Inhibition of RPE cell phagocytosis by white light exposure. At 48 h after white light exposure, ARPE-19 cells were incubated for 2 h at 37 °C in culture medium with 100 μL of the prepared pHrodo™ BioParticles® suspension. Measurements of fluorescence were taken by using a multiwell spectrophotometer. The ability of phagocytosis of ARPE-19 cells was decreased 0.6-fold.

real-time RT-PCR. As expected, the mRNA of α-SMA and vimentin increased 2.5–4-fold and that of ZO-1 decreased approximately 0.8-fold at 48 h after white light exposure (Fig. 3). Western blot analyses were also performed to examine the protein levels of vimentin, α-SMA and ZO-1, after the exposure of white light for 48 h. These blots confirmed the increased expression of vimentin, α-SMA and the decreased expression of ZO-1 (Fig. 3). Immunohistochemistry was also performed, revealing the evident up-regulation of vimentin expression (Fig. 3). In normal state, expression of ZO-1 was detected at the cell membrane. After the light exposure, the regular expression of ZO-1 at the cellular membrane was disrupted.

Inhibition of RPE cell phagocytosis by white light exposure

It is conceivable that the RPE cells that lost the expression of epithelial markers are functionally impaired. Thus, the results led us to investigate the function of RPE cells after white light exposure. To determine the effect of white light exposure on the phagocytotic ability of ARPE-19 cells, phagocytosis assay was performed using Bio Particle. The results demonstrated that the ability of phagocytosis of ARPE-19 cells decreased 0.6 fold after light exposure for 48 h (Fig. 4).

Discussion

The major finding in this study was the exposure to white light leads to the activation of β-catenin in RPE cells. Specifically, in this study, it was demonstrated by Western blot analysis that the expression of phosphorylation of β-catenin decreased and the translocation of β-catenin was observed by immunofluorescence after white light exposure using ARPE-19 cells. Recently several groups have revealed that oxidative stress modulates the function of β-catenin. It has been demonstrated that treatment with H_2O_2-induced stabilization of β-catenin and an increase in the expression of endogenous Wnt target genes using murine fibroblast-derived cell line [11]. Another group has shown that redox-regulation contributed to modulation of cell signaling pathways that lead to endothelial barrier dysfunction using bovine lung microvascular endothelial cells [20]. Furthermore, it has been demonstrated that oxidative stress leads to disruption of the cadherin-β-catenin complex, leading to the activation of β-catenin signaling pathway [21]. In RPE cells, visible light is absorbed by endogenous photosensitizers such as cytochrome c oxygenase and lipofuscin, and produces reactive oxygen species [5]. The current study did not support that the presence or absence of a major lipofuscin component, A2E, affects the light-induced effects of β-catenin. Thus, the current results support that the oxidative stress is induced by white light exposure-activated β-catenin. The light source used in the current study had a peak of approximately 420 nm and the light output is mostly short wavelength light. Whether this blue portion of the visible light is most effective in inducing β-catenin activation, as shown in previous studies investigating the effects of visible light on RPE cells [5] awaits further study.

Another important finding is that the white light exposure leads to the differential expression of epithelial and mesenchymal markers in RPE cells. EMT of the RPE cells occurs in several pathologic conditions and is most typically observed in pathologic conditions such as proliferative vitreoretinopathy (PVR). Migration of fibroblastic RPE cells expressing the mesenchymal cell markers proliferate along the vitreo-retinal surface and massive proliferation contributes to the formation of PVR [17]. RPE cells that express mesenchymal markers can be found in the choroidal neovascular membrane due to AMD [22–25]. Furthermore, integrity of RPE cells is disrupted in the early stage of AMD without choroidal neovascularization [26], and mice lacking SOD2 exhibit similar lesions to those observed in AMD. It has also been noted that the ZO-1 expression decreased in RPE cells [13]. The current study demonstrated that the expression of vimentin and α-SMA were increased and the expression of ZO-1 was decreased after white light exposure in ARPE-19 cells. It has been reported that EMT occurs in vitro in the ARPE-19 cell in response to growth factor(s) [17]. Another group has reported that hepatocyte growth factor-induced β-catenin signaling pathway is involved in the phenotypic change of RPE cells, and leads to RPE migration [27]. The present results, in conjunction with those of previous investigators, support that external stimuli induce the loss of epithelial markers and acquisition of mesenchymal markers in RPE cells. In the current study, cellular proliferation of RPE cells was not affected after the cells were exposed to the white light.

With ageing, chronic oxidative stress causes RPE cell dysfunctions that are believed to be central in the development of AMD. Thus, it is important to understand how sub-lethal oxidative stress affects RPE function. The current results support that the oxidative stress induced by white light exposure-activated β-catenin and lead to loss of epithelial markers and acquisition of mesenchymal markers in RPE cells. Concomitantly, the ability of phagocytosis of ARPE-19 cells decreased after light exposure. A previous group has shown that activation of α2 AMP-activated protein kinase contributed to the inhibition of RPE cell phagocytosis by oxidative stress [28]. Further molecular analysis of the light exposure on the RPE cell phagocytosis is an important issue for future research.

In conclusion, the effects of sub-lethal white light exposure on RPE functions was investigated. The results indicated that the exposure of white light activates the β-catenin pathway, leading to the loss of epithelial markers, the gain of mesenchymal markers and the impairment of the phagocytosis ability of RPE cells. The current study demonstrated the effects of prolonged relatively intensive white light exposure on RPE cell function, and suggests that visible light exposure leads to impaired RPE cellular function. While whether longer and chronic light exposure exerts such effect on RPE cells in environmental circumstances awaits further study, the current study does support a link between the β-catenin activation by white-light and AMD.

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